

No end to nuclear decay?

The stagnation of the nuclear power industry in the United States should be an incentive for radical thinking rather than an excuse for despair.

THE predicament of the nuclear power industry in the United States will get worse before it gets better. Last July, nuclear power provided the occasion for the biggest municipal default in history, when the Washington Public Power Supply System failed to meet its payments on bonds worth more than \$2,000 million. More financial embarrassments are in store. It appears to be only a matter of time, for example, before the Long Island Lighting Company (LILCO) concedes that its Shoreham plant, which is already a decade behind schedule and seems likely to cost 15 times more than intended, will never be allowed to operate. LILCO's chief executive, Dr William Catacosinos, admitted for the first time last week that it might be simpler for the utility to abandon Shoreham than to continue its enervating struggle with the local municipality, Suffolk County. Suffolk, it will be remembered, has done its utmost to thwart LILCO's plans for Long Island by refusing to participate in the emergency evacuation planning mandated by the Nuclear Regulatory Commission (NRC).

Shoreham's plight is a depressingly familiar (but exceptionally acute) compound of engineering, economic and political failures, failures that have contributed to the fact that there has not been a single new order for a nuclear reactor in the United States since 1978. The nuclear industry, supported by the Reagan Administration, lays much of the blame at the door of NRC. Less cumbersome procedures, fewer opportunities for public intervention and a limit on the number of design "backfits" ordered by NRC could, it is suggested, transform the economics of reactor construction. But there is little point in looking for relief in that direction. Congress, backed by public opinion (half of the public is opposed to building nuclear plants, a third supports it), is in no mood to reduce the powers of NRC, a body which is criticized for too cosy a relationship with the industry at least as often as it is accused of over-zealous enforcement of its rules. There may indeed be a case for strengthening NRC procedures in order to revive public confidence in the safety of nuclear energy.

It is in any case far from clear that regulatory reform by itself would do much to alter the predicament of the industry. The two main causes of that predicament are the abrupt fall in the rate of growth in electricity demand since 1973 and the simultaneous increase in the costs of nuclear reactor construction. Between 1960 and 1972, electric utilities took for granted annual growth rates of 7 per cent, and it is on those assumptions that existing nuclear power construction was predicted. In practice, growth has averaged only 2.5 per cent a year since 1973. Moreover, the costs of building nuclear plants doubled in constant dollars during the 1970s and are expected to increase by another 80 per cent for plants now under construction. Some of this increase can be ascribed to new regulatory requirements but not much, according to a new study, *Nuclear Power in an Age of Uncertainty*, published by the Office of Technology Assessment (OTA) last week. OTA points out that of the group of plants now being built, the most expensive is expected to cost more than four times as much as the least expensive. The variation has something to do with regional labour costs and differences in weather but is primarily a result of differences in the competence and experience of management.

So it is to its own management, rather than to external agencies, that the nuclear industry should look for a way out of the impasse in which it finds itself. Unfortunately, industry managers show

little enthusiasm for tackling any of the major structural problems that have to be solved. There is a temptation instead to ride out the hiatus in orders for new plants in the hope that demand for nuclear power will revive when the demand for electricity revives. Meanwhile there is a reasonable living to be made by servicing existing plants and, from time to time, selling reactors overseas.

That strategy might work, if the hoped-for recovery were to come within five years. But if the hiatus lasts a decade or more, the prospects for reviving the industry are much less promising. According to OTA, the lack of orders for new plants is already creating shortages of spare parts and personnel. Enrolment for nuclear engineering degrees has declined since the mid-1970s, and graduate numbers will barely be enough to fill the anticipated need for 6,000 engineers to operate plants by 1991. Instead of alternately sitting on its hands and wringing them, the industry should be acting now to ensure that the hiatus ends sooner rather than later.

What can be done? For a start, the industry could stop talking about standardized designs and actually begin to standardize them. Without some degree of standardization, as in France, construction of nuclear reactors is unlikely to be able to compete with coal-fired plants. Standardization would require ruthless surgery to reduce the proliferation of vendors and designers, but that would be better than seeing the whole industry gradually collapse under the weight of its own diversity. Second, the industry could begin to address some of the concerns of its critics, by taking more visible measures to ensure competent management and to enhance safety standards. Why not look seriously at alternatives to the much-criticized light-water reactors — at high-temperature gas-cooled reactors or the Swedish PIUS (process inherent ultimately safe) reactors, in which the core and other critical reactor components are submerged in a pool of water? □

How to help Orlov

Some of the schemes being considered in the West on behalf of Yuri Orlov will not work.

SEVEN years ago, the Soviet accelerator physicist, Yuri Orlov, was arrested in Moscow and charged with making anti-Soviet propaganda. At his trial, Orlov was sentenced to seven years in gaol followed by five years of exile. During the first part of his spell in prison, Orlov seems to have been a thorn in the flesh of the prison authorities, protesting at censorship of mail and the molestation of prisoners by their guards. During this period, he seems also to have kept his hand in with his physics. More recently Orlov seems to have been ill, perhaps gravely so. Orlov's case, like Sakharov's, has become to many in the West a test of Soviet attitudes towards professional people who happen to disagree with government policy. Excitement has come to a head (see page 585) now that the more rigorous part of Orlov's sentence is at an end. Will he be released into exile, perhaps abroad, or cynically be kept in gaol?

Orlov was the chief organizer of the group active in Moscow in the mid-1970s in the monitoring within the Soviet Union of the Helsinki agreements. This formless group of undertakings commits its signatories, among other things, to permit the free



flow of ideas and even people between themselves. Orlov and company have plenty to complain about.

There have been several well-publicized international shouting matches, most recently at Madrid, but the agreements have also led to the Stockholm talks on European security, now under way, which may yet make ten years of diplomacy seem worthwhile. In any case, Orlov was not imprisoned for having discovered that the Helsinki agreements were being illiberally interpreted (more simply, ignored) by Soviet authorities, but for having said so, and for having distributed his complaints abroad.

By that test, Orlov is unquestionably a criminal. Did not a Soviet court, whose judicial independence is guaranteed by the Soviet constitution, come to that conclusion? Is it not, in any case, well known that the Soviet authorities, like their tsarist predecessors, have always taken a serious view of what seems to be disloyalty? With the obligations of individuals to the state counting for more than the state's obligation towards its people as individuals, Orlov must have known that he was asking for trouble. Orlov's activities had much in common with those of earlier trouble-makers. Medvedev, Solzhenitsyn and the like find some weakness in the system, such as the constitutional right that private correspondence should not be interfered with, exploit it to the full and, provided they keep within the law, sooner or later find themselves at an airport with an exit visa in their pocket. Orlov must have overstepped the line.

This is how Orlov's case seems to many honest Soviet citizens. People elsewhere distressed at Orlov's plight must take that disconcerting circumstance into their calculations. Two conclusions follow. First, on compassionate grounds, anything that will help to release Orlov from the plight in which he finds himself is to be welcomed. If the petition signed by 2,600 French physicists, or the proposal being canvassed among the accelerator people at Geneva that Orlov should be offered a job at the European Organization for Nuclear Research (CERN), will help towards that end, all well and good. But the chances are not high. That is why this sad occasion should be a reminder to complainants in the West that their quarrel with the Soviet authorities goes deeper than the circumstances of Orlov's imprisonment and is tantamount to a protest against Soviet law and the manner in which it is interpreted. But sovereign governments do not often change their legislation at the behest of people who live elsewhere. So, for the long haul, the complainants must find subtle ways of making their opinions felt. The proposal, put forward last week, that there should be an Orlov Prize to honour people like him, will not help and may even hinder. Breaking off such personal scientific collaborations with Soviet scientists as have survived the past few years would be similarly nugatory. It will be better to keep the links going and to use them as a means of convincing collaborators that there are good reasons why the law needs changing. Orlov's objective, after all, is that his own brave act should ensure that there is less need of an army of successors. □

What hope for Esprit?

The European plan for information technology research is likely to be launched too late.

THE European Communities are more conspicuously than usual travelling in cloud-cuckoo land. Last week, the Commission, the Communities' executive authority, put the finishing touches to its plan for supporting long-term research in information technology by means of an intricate system of research contracts to be let by Brussels to companies and other research organizations which are themselves willing to meet half the cost.

The European strategic programme of research and development in information technology (known as Esprit) has recently been nurtured by Etienne Davignon, the Commissioner for Industry in Brussels, who has been shuttling about Europe seeking promises of support. On Monday, Davignon spent 50 minutes at 10 Downing Street with the British Prime Minister; the hope now is that if British objections can be overcome, the project

will be approved at a meeting of ministers on 28 February, whereupon the Commission will be free to spend roughly US\$400 million sterling of its own money provided that its partners commit an equal sum over the succeeding five years. The drawback, for the time being set aside in Brussels, is that the Commission is certain on present form to run out of funds between June and September. In short, the Commission has no money for projects like Esprit, and will not know whether it will be in funds again until the meeting of the heads of European governments fixed for 20 and 21 March. On recent form, the chances that the Communities' deep-seated differences can be resolved at a single meeting, and before the last minute has arrived (which will not be until June), are negligible.

So will Esprit be another European dream impaled on the failure of European governments to agree what should be done with their common enterprise? If it is, it will not be the first casualty of this kind. In the 1950s, Euratom, then legally a community in its own right, embarked on an ambitious programme for the development of nuclear reactors whose hallmark was that their design had nothing in common with those of the reactors being developed in member states. Member governments would not countenance the threat of competition between their own national nuclear energy establishments and the common enterprise, but the result was what everybody would have been entitled to predict: Euratom's projects failed (as did many of those mounted nationally). The idea that there might be a European project to strengthen the technology of the computer and communications industry was the most prominent of the proposals canvassed in the late 1960s by M. Pierre Aigrain, then President Pompidou's chief scientist. That came to grief for two reasons — the resistance of European telecommunications monopolies to the suggestion that their own research was insufficient and the lack of a plan to suggest how a common research programme supported by established companies or nationalized industries could nevertheless yield benefits that would be widely shared. Aigrain's project came too soon.

The Commission's latest attempt to breathe life into European information technology has obviously been designed to avoid these pitfalls, but has weaknesses of its own. Participation in the programme will be voluntary, but collaborating companies or consortia will have to commit resources to projects that the Commission supports. This will ensure a degree of seriousness. There are also rules about the availability of the results of research and development projects although, inevitably and even equitably, the partners in the research projects will have an advantage over others in their exploitation deriving from familiarity with the work. Perhaps the most encouraging feature of the Esprit programme is that it seems already to have fostered a degree of collaboration between European companies hitherto not much used to talking to each other. But the question remains of whether Esprit is what Europe most needs.

That information technology offers great opportunities for European industry, and that technology of the kind now being applied is likely to seem, a few decades from now, a rudimentary tool, are nowhere denied. Most industrial organizations recognize which way the wind is blowing. But the impediments to faster development consist not so much of the lack of development projects as of the shortage of people able to turn bright ideas into working machines decisively and quickly, the lack of technical people able to service the machines actually sold to customers (which demands more skill than that required to order a package of software from a list) and too little support for really long-term projects, those that might lead to optical computers for example (see *Nature* 9 February, p.494). For all the delicacy with which Esprit has been conceived, the programme will help towards these ends only incidentally, by training people. Might not the same result have been obtained by other means? (The same criticism can be made of the British Government's Alvey programme.) The risk in what the Commission is proposing is that a great deal of money (which does not exist) may be spent ineffectually. The danger is that if the Commission fails with this huge project, it may not be given another chance. □

European community

French visions of Europe and space

At last, a French phoenix has arisen from the ashes of the European summit at Athens last December. In Athens, President François Mitterrand of France apparently put paid to all hopes of negotiation with Britain over the future of the European Community. In The Hague last week, he explained his action: not directly, but by presenting a technological vision of a new Europe, in which the old conflicts would be something of a past age.

One element of this future would be a European space station, as a military observation post in the first place, but one that could also carry laser and other star wars weaponry for the defence of Europe.

Meanwhile, M. Mitterrand proposed only partial solutions to Europe's overriding political problem (the European budget), solutions which are unlikely to find favour with the crusading British Prime Minister, Mrs Margaret Thatcher.

Surprisingly, one of M. Mitterrand's most favoured ministers, M. Laurent Fabius (industry and research) seemed last week to have been caught a little un-

perhaps more blinding — the worse becomes France's international and internal position. (Internally, Mitterrand is facing rioting in factories against his restructuring policies, and a probable rout at the European Parliament polls in the summer.)

Putting it boldly, the Mitterrand vision is this: the European Community is out of date — and so are Europe's defence arrangements with the United States. So why not try for something completely new?

The European Community, for example, is based on post-war treaties which saw iron, steel, coal and nuclear as the key economic technologies. Now the key technologies are electronics, informatics, aerospace and biotechnology. The Community must be based on these, says M. Mitterrand, echoed by Fabius at Toulouse.

Equally, M. Mitterrand is disturbed by President Reagan's attitude to Europe, both technological and military, and — with France outside the North Atlantic

Treaty Organization — he feels freer than most in Europe to propose something approaching an independent nuclear defence: hence the European space station.

Here, however, M. Mitterrand mixes military and industrial motives. While CNES has not admitted to a military space station study, it has since 1981 been studying a non-military one (dubbed Solaris), along with a recoverable low-orbit injector (Hermes) looking much like the space shuttle. Clearly, the object was to give Europe the possibility of recovery, repair, refuelling, construction and other options in space that the shuttle gives the United States but that Ariane has not given Europe (Ariane 5 — already on the drawing board — could launch a future Hermes).

In M. Mitterrand's mind at The Hague, these essentially commercial questions of Europe's industrial place in space in the 1990s were being mixed with other issues, but his overall dream was clearly of a brave new European Community based on brave new ideas.

Unfortunately, neither Mitterrand nor Fabius last week had much to say about mundane things such as the price of milk and the beef mountain, which may cause the French vision to become a mirage.

Robert Walgate

UN biotechnology

Let a hundred labs bloom

New Delhi

THE contentious issue of the location of the International Centre for Genetic Engineering and Biotechnology for developing countries, sponsored by the United Nations Industrial Development Organization (UNIDO), seems to have been resolved. Although the original intention was to set up the institute in a developing country, the preparatory committee for the centre has now recommended that it is split into two components to be established in New Delhi, India, and Trieste, Italy.

The committee, meeting in Vienna at the end of January, suggested that the New Delhi component should concentrate on research in agriculture and human and animal health and that the Trieste laboratory should emphasize industrial microbiology and energy research. The offers from the two countries have been found to be most attractive.

A high-level meeting of the national representatives on 3 April will in all probability approve this decision, reached by the preparatory committee of 27 member countries. The committee was set up after the abortive Madrid meeting in September last year failed to decide on a location, following which more than one centre seemed a necessary compromise.

The decision reflects a major victory for India in the teeth of opposition from the UNIDO scientific committee which had favoured siting the centre in Belgium, Thailand or Italy. The committee said that

these countries had sufficient merits to offer good prospects for the foundation of the international centre. India, like Cuba and Pakistan, had been ruled out on the grounds that its background in basic science is insufficient and that it would be difficult to attract good scientists.

This argument has now been rejected. What ultimately clinched the issue in favour of India was the specific outline of the offer involving some US \$19 million. The offer includes land, buildings and laboratories; technical and auxiliary staff; permanent equipment and \$5 million to cover recurring costs for running the centre for five years.

The government has promised that the New Delhi centre will have "high standards of scientific and laboratory facilities dedicated to solving problems relevant to developing countries in a highly focused and multidisciplinary approach". It will also provide training to scientific and technical personnel from developing countries.

The preparatory committee also tries not to displease the other nations that had also staked their claim to host the international centre. They can set up their own units affiliated with the New Delhi and Trieste centres and funds for them will also be provided through international sources. These affiliated units can be converted into centres by the board of governors of the new centre following a review after three years of operation by the New Delhi and Trieste Laboratories.

Sunil Saraf



prepared by his president's commitment to a space station. Speaking at a meeting of European Parliament socialists in Toulouse, in south-west France, Fabius described the station as having commercial, scientific and strategic purposes — with, it was implied, the "strategic" element firmly relegated to third place. The French space agency CNES had been studying the possibility of such a station for a few months, said an aide to M. Fabius, although the CNES director-general had earlier denied it.

Certainly, President Mitterrand has been taking an increasingly independent approach to European policy lately, so much so that French officials have occasionally been seen to be announcing conflicting policies. This apparent chaos, however, is probably the consequence of Mitterrand's commitment to a vision which becomes more dazzling — and

Recombinant DNA

Watch on human experiments

Washington

THE Recombinant DNA Advisory Committee (RAC) in the United States last week reaffirmed its intention to review any experiments involving human subjects or the release of recombinant organisms into the environment. Under rule changes adopted overwhelmingly by the committee, any proposal to transfer recombinant DNA into human subjects would have to be cleared by the full RAC.

Although the rules are legally binding only upon recipients of federal research money, industry and all government agencies have agreed voluntarily to follow the RAC procedures.

RAC's decision formally to require a review of human genetic engineering experiments was prompted largely by the demise last year of the President's Commission on Bioethics and the resulting concern that no group was monitoring developments in this field. RAC also decided to establish a nine-member working group, made up of scientists, lawyers and ethicists, to look at any human genetic engineering proposals. This move parallels a legislative proposal by Representative Albert Gore (Democrat, Tennessee) that would create a human genetic engineering commission with similarly broad representation. That proposal passed the House last year, but was never enacted into law. RAC itself is dominated by scientists and physicians.

In a related discussion of the scope of RAC's oversight, RAC appeared committed to continuing to review deliberate environmental release experiments and industrial activities. The Food and Drug Administration and the Department of Agriculture offered strong support for RAC's role in these areas; the Environmental Protection Agency (EPA), which has announced its intention eventually to regulate deliberate release of recombinant organisms under the Toxic Substances Control Act, said at last week's RAC meeting that it supported RAC's handling these areas at least until EPA's regulations are issued. A report released last week by the staff of Representative Gore's subcommittee questioned whether RAC has the expertise or the administrative capacity to handle a large flow of proposals from industry.

Anti-genetic-engineering activist Jeremy Rifkin was much in evidence at last week's meeting, notably for obtaining a court order barring RAC from holding a closed session to discuss a deliberate-release experiment proposed by Advanced Genetic Systems, Inc (AGS). The company is the sponsor of the planned release of frost-resistant bacteria by Steven Lindow of the University of California, Berkeley, currently tied up in other litigation initiated by Rifkin. AGS decided to submit to RAC its own proposal to conduct a similar

experiment. In accordance with standard procedures for dealing with proprietary information, RAC announced a closed session to review the proposal.

At the behest of several RAC members, a decision was later made to open as much of the discussion as possible. Rifkin argued in his lawsuit that RAC did not have sufficient grounds to close any of its discussions of the proposal and that the subsequent decision to open part of it was not announced 30 days in advance in the Federal Register, as required for public meetings. A US Court of Appeals panel, acting on the technicalities of Rifkin's complaint rather than its substance, ordered RAC not to hold its discussion until it had published proper advance notice and had it explained why any portion of the discussion should be closed to the public.

Rifkin also appeared at the meeting to protest against a proposal from researchers at Uniformed Services University of the Health Sciences to lower the containment required for a previously-approved experiment aimed at cloning the gene of a bacterial toxin responsible for dysentery. Rifkin, claiming the support of several prominent arms-control experts, said the experiments should not commence until an "Arms Control Impact Statement" was prepared on the research. The Arms Control and Disarmament Agency is charged by Congress with preparing such a statement on any government research with potential military applications.

Rifkin said these experiments could be used to create biological warfare agents, and that RAC members "would be personally liable under international law and the principles enunciated at Nuremberg in aiding the commission of crimes against humanity" should the work lead to biological weapon development.

Paul Warnke, former SALT negotiator and the first name on the list of supporters claimed by Rifkin, said in an interview that he had not endorsed Rifkin's remarks, but had only told Rifkin in a telephone conversation that he believed an impact statement would be important in judging the implications of the research. He said that he neither supported nor opposed the actual research.

Proponents of the experiment, which was approved by RAC last year at P4 containment, said the research was vital to the development of a vaccine against the leading cause of dysentery. RAC approved the request to lower the containment requirement to P2 by a 9 to 5 vote with 4 abstentions. The director of the National Institute of Allergy and Infectious Diseases, to whom RAC reports, has generally declined to accept recommendations made on split votes such as this one.

Stephen Budiansky

Australian technology

Tax perks the stimulus

Canberra

AUSTRALIAN investors and technologists are likely to benefit from the Management and Investment Companies Act that came into force at the beginning of this month. The act embodies a tax-deductible venture capital scheme designed to bring together investors, innovative small businesses and management and investment companies (MICs). A licensing board, appointed by Mr Barry Jones, Minister for Science and Technology, is to select about eight MICs from the large number of expected applicants and to approve the amount of tax-free capital each can raise.

The MICs will choose several small, local high-technology businesses which they believe can sustain an annual sales growth rate higher than 20 per cent over the next three years. They will supply the businesses with long-term equity, loan capital and entrepreneurial advice, especially in marketing and finance. The source of the venture capital supply to the MICs will be investors who may claim 100 per cent tax deduction on the capital subscribed in the year of the investment.

Like Britain, Australia has done poorly in bringing technological innovation to commercial success: the technologists blame the businessman's conservatism and financiers blame the inventor's lack of management skill. The MIC scheme is based on recommendations of the Espie committee, set up by the previous government to investigate ways of financing high-technology enterprise in Australia. The committee perceived a gap at the high-risk, high-return end of the Australian capital market. The scheme may go some way towards plugging it. As if to fend off the possible jibe that a business with sales expanding at more than 20 per cent a year needs no special assistance and that the gap is merely the embodiment of Australian lack of self-confidence, Mr Jones recently echoed the Espie committee's remark that it "knew of no country which had succeeded in establishing a climate for investment in high technology without the government taking positive action".

The first MIC licences are expected to be granted in April. Over the first five-year period of phased capital injections into the ventures, \$A200 million will be licensed for subscription to the MICs, at an estimated tax revenue loss of \$A100 million.

On 24 January, the Treasury altered the proposed tax-incentive schedule for investors to promote long-term "patient" equity investment in the MICs and to inhibit abuse of the scheme. Investment for less than two years now attracts no net tax deduction; for the full, 100 per cent deduction, a four-year investment is necessary.

Jeffrey Sellar

Soviet Union

Orlov's fate in balance

DR Yurii Orlov, the Moscow physicist and human rights campaigner, last week completed a seven-year prison sentence for slandering the Soviet system. Ironically, the expiry of his term (on 10 February), coincided with the announcement of the death of the Soviet leader, Mr Yuri Andropov, who at the time of Orlov's trial was head of the secret police (KGB). Dr Orlov is now due to serve a further five years in Siberian exile. Last week, however, his supporters in London and Geneva announced proposals aimed at ameliorating the remainder of his sentence.

At his trial, Dr Orlov openly admitted charges of circulating and distributing to the Western press documents alleging breaches of the Helsinki accords and international human rights conventions within the Soviet Union. The basis of the prosecution case was that these allegations were false. However, Mr John Macdonald, who failed in his attempts to travel to Moscow to serve as Orlov's defence lawyer, still maintains that they were true, and last week, at a press conference held at the Institute of Physics in London, announced his intention of petitioning the Soviet courts to reopen the case on the grounds that much pertinent defence evidence was never considered. Such a petition has little chance of succeeding because Mr Macdonald's proposed witnesses are either citizens of Western countries or else recent emigrants from the Soviet Union — people whose motives would be suspect from the Soviet point of view.

A more realistic proposal is a petition that Dr Orlov's "internal exile" be commuted into "external exile", that is, that he be sent abroad. One suggestion is that he could be offered a job at the European Organisation for Nuclear Research (CERN) with the LEP (large electron-positron) storage ring project. The governments of the thirteen member-states of CERN have been unofficially canvassed, and most of the replies so far received have been cautiously supportive. CERN has, incidentally, a long-term cooperation agreement with the Soviet Union which was extended six months ago, and which includes work on LEP.

As to Orlov's scientific fitness for such a job, this was beyond dispute before his imprisonment, and it is known that he has tried to keep up his scientific work while in the prison camp, although, after a few early letters (an extract from one was reproduced in facsimile in *Nature* 277, 591; 1979) the camp censors clamped down on anything resembling a scientific equation for fear it could be a coded message. If he is, indeed, sent to Siberia, Mr Macdonald has suggested that scientists from CERN should apply for visas to visit him — and if refused should consider some form of "non-cooperation" with the Soviet Union.

CERN has a vigorous "Orlov Committee" whose activities have included defence of scientists in Uruguay, Turkey, Morocco and Poland as well as the Soviet Union, mustering on occasion over 600 signatures out of a scientific workforce of 3,500. This committee supported Mr Macdonald's proposal but stressed that each individual must decide for himself the "appropriate" response to a refusal. (Ethics apart, it would make no sense for one solitary member of a large team to opt out of a joint project.)

Finally, Mr Macdonald suggested the establishment of a \$50,000 annual "Orlov

prize" for the defence of human rights, for which he would approach the Rockefeller Foundation in the United States for funds. The CERN Orlov Committee again approved the idea in principle but suggested that it should be funded either by a consortium of European universities or by neutral governments such as Sweden and Switzerland, since the backing of these bodies would disarm possible Soviet criticism that the prize was simply a capitalist propaganda ploy.

At the time of writing it is uncertain whether Dr Orlov has been released from prison. Soviet legislation provides for an extension of sentence if prison rules are contravened and Dr Orlov has already lost visiting and correspondence rights for alleged contraventions.

Vera Rich

West German universities

The price of protest

Bublingen, West Germany

A PROTEST in West Berlin last week by 90 university lecturers against the siting of Cruise and Pershing missiles in West Germany, in defiance of instructions from the presidents of the Free University and the Technical University, was echoed at a conference here organized by the anti-*Berufsverbot* campaign. *Berufsverbot* — the exclusion from employment in the government service of those whose political views are deemed to present a threat to the state — has been practised in West Germany since the early 1970s.

The procedure is deemed by many constitutional lawyers to be contrary to the guarantees in the West German constitution of civil liberties and freedom of conscience. The practice was authorized by a special decree of the federal government, and has never gone through the parliamentary law-making procedure. Its implementation varies in the different *Länder*, and in Hesse, where the Greens hold the deciding vote in a hung parliament, they are pressing for the practice to be dropped altogether.

The new development since the last anti-*Berufsverbot* conference in Hannover in 1982, the campaigners say, is that restrictive measures have been increasingly employed against peace campaigners. There were complaints that the practice of *Berufsverbot* has been supplemented by restrictions on demonstrations and penalties for those taking part on the grounds of public order.

Surprisingly, and in contrast with previous conferences, there was little to report this year from the university sector. University lecturers and schoolteachers, being paid by the state, are considered to be "public servants". One view is that all lecturers at risk have already been sacked, while new graduates with doubtful political views are unable to gain university employment.

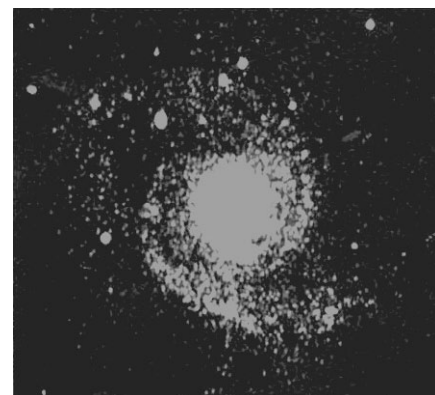
The only striking new *Berufsverbot* case

affecting the universities reported at Bublingen was that of Hans Joachim Wunderlich, a mathematician who was dismissed last year from the Karlsruhe University Computer Centre on the grounds that he constituted a security risk, since the computer was also leased for defence-related industrial research. So far, university staff taking part in peace activities have not been penalized. This contrasts sharply with school teachers, many of whom are now facing disciplinary proceedings for taking part in the "five-minute strike" for peace organized by the Trade Union Federation (DGB) on 5 October 1983, or for allegedly "over-emphasizing" the theme of peace in school projects and concerts.

The universities have not been directly affected by the "Strike for Peace" because it took place on a Saturday (in West Germany a working day for schools but not for universities). The Berlin protest may be different.

Vera Rich

La Palma's Newton



THE Whirlpool Nebula (M51) as seen by the United Kingdom's 2.5-m Isaac Newton Telescope, newly erected at the La Palma Observatory in the Canary Islands. The official "First Light" was received by the telescope on 13 February.

Remote sensing/mapping

Call for UK investment

THE remote sensing market is an essential area for UK involvement, according to a report on *Remote Sensing and Digital Mapping* (HMSO, £7) produced last week by the House of Lords Select Committee on Science and Technology. And while the government is asked to increase investment in selected areas, most of the report's 52 recommendations concern changes in the infrastructure of the remote sensing and mapping communities. In particular, there is a call for greater effort in the handling and interpretation of digital data and the development of a network of linked computers in key centres.

The committee highlights the market potential of remote sensing, especially the current investment in the United States and France in satellite imaging systems. A major recommendation is that the National Remote Sensing Centre at the Royal Aircraft Establishment, Farnborough, should be given more staff and become the central "executive" node of a national computer network — a role similar to that of the Rutherford Appleton Laboratory in the astronomers' 'Starlink' network.

The aim would be to enhance the general accessibility of remote sensing data. The committee emphasizes that a significant challenge for the future will come not so much from advances in satellite instrumentation (although increased spectral resolution and radar sensitivity are two key areas of research) but through the sheer volume of data that such technical advances will produce. Two areas of strength, therefore, on which the United Kingdom should build are remote sensing radar and software development.

On satellite vehicles themselves, the committee advocates enthusiastic participation in European Space Agency projects such as ERS-1 — a remote sensing satellite due for launch in 1986 — but also bilateral collaborations, particularly with the United States and Canada.

Another important recommendation is that there should be a formal link between the research councils in the form of a joint committee for basic research. This is likely to be welcomed by those working on the proposed Satellite Radar Altimetry project (see *Nature* 306, 108; 1983) now under consideration by the Natural Environment Research Council and the Science and Engineering Research Council. The project is not only costly (requiring about £18

million over ten years) but also falls within both councils' areas of responsibility, and, as a result, has encountered difficulties. Yet its supporters argue that it would incorporate the development of just the sort of expertise in instrumentation and data-handling that the House of Lords committee seeks.

Philip Campbell

● The report also concludes that the Ordnance Survey should increase its qualified research and development staff if it is to maintain its leading role among British mapping agencies and that the survey's digitization programme should be speeded up.

These conclusions will be as music to the ears of Ordnance Survey staff, who have been facing an uncertain future. Formally, the survey is a government department financed on a vote from central funds. A plan last year to re-establish it as a "trading fund" met with a chorus of protest from a variety of Ordnance Survey users, and was defeated in the House of Lords. Last month Mr Patrick Jenkin, Secretary of State for the Environment, announced that the survey will remain on voted funds but will be required to work to clearly defined financial targets.

The digital mapping programme is very much in its infancy but the committee believes — and some at the survey are ready to agree — that demand for digital maps is likely to increase because they can be displayed or printed very rapidly, to a variable scale and with user-defined edges. Moreover, particular features can be emphasized, or omitted for clarity, and digital maps are easily updated.

The committee wants a reversal of the recent "insensitively applied" manpower cuts of almost 25 per cent in five years. As a second best, even more of the digitizing work should be contracted out than at present, to make savings which would then pay for extra staff. In this way, the Ordnance Survey could become a national centre for digital mapping incorporating a development team headed by a chief scientist.

But the House of Lords committee does see some difficulties on the horizon. Perhaps the most important is that of copyright. For many years, some small societies, such as the Chiltern Society, have been selling their own annotated versions of Ordnance Survey maps. But, in keeping with its new business-like image, the survey will not allow the sale of such competing maps in areas where it has produced an adequately detailed series of its own. With digital maps the problems may be much more difficult: the Ordnance Survey supplies base data from which any number of maps may be produced and which may be continually updated. Their Lordships decline to offer a solution to the problem.

Tim Beardsley

Czechoslovak science

Large gap to bridge

PLANNERS in Czechoslovakia must reject slogans about the "omnipotence" of science and technology, since these can foster "dangerous illusions", deputy federal premier Jaromir Obzina warned last month. He was addressing the first meeting of the new State Commission for Scientific, Technical and Industrial Development, set up to close the gap between research and development on the one hand and the practical implementation of results on the other.

Before the establishment of the Czechoslovak commission, there had been considerable discussion in the press about the reasons for the gap. The general opinion was that there is no serious lack of funds for research — indeed, some allocations are not taken up or spent in full. Instead, the main cause appears to lie in the personalities of those responsible for implementing the results, a view reiterated by Mr Obzina in his reference before the commission to "insufficient coordination" between research and production and to "serious shortcomings" in management.

The purpose of the commission, Mr Obzina said, is to reveal the real causes of the shortcomings, and to deal with real problems. Energy should not be wasted on theoretical "pseudo-problems" and discussions, "however brilliant and witty they might be". But the task, he implied, is likely to be a long haul. The process of "socialist industrialization" may be compared with collectivization in agriculture — "a programme of persistent work for a number of decades".

He warned the commission against associating itself with "naïve people" who "today enthusiastically applaud scientific and technical progress", but whose personalities lack "persistence, diligence, perseverance and selflessness". Such people, he said, will be all too likely to turn against science when they find that "no cheap results have fallen into their laps".

Such remarks must, of course, be politically irreproachable, coming as they do from a deputy federal premier with the endorsement of party presidium member Milos Jakes, who pledged to the commission the "effective support" of the party "at all levels". In another context, and from a less eminent speaker, they might be construed as containing a certain veiled criticism not only of Marxism (which bases its validity on its "scientific" nature) but also of the Soviet Union where, during the later years of the Brezhnev era, there was undoubtedly a tendency to expect, in Obzina's words, "some kind of miracle" as a result of that continuously repeated slogan "accelerated application of scientific and technical research findings in practice".

Vera Rich

Correction

SIR Peter Hirsch's share of the 1983–84 Wolf Foundation prize in physics was for his contributions to the utilization of the transmission electron microscope and not the scanning electron microscope as claimed in *Nature* 2 February, p.407. □

Nuclear waste

UK procedures condemned

THE Nuclear Installations Inspectorate (NII) and the Department of the Environment (DoE) this week publish separate, highly critical reports of safety procedures at the British Nuclear Fuels Limited (BNFL) reprocessing plant at Sellafield (formerly Windscale). The reports investigate incidents at the plant last November that led to widespread radioactive contamination and a public warning from the Department of the Environment against the use of beaches in the area.

BNFL's latest problems began on 11 November, when operators pumped wastes into a sea discharge tank, apparently unaware that radioactive solvent and "crud" had not first been separated off. Standing instructions for the procedure, which was being undertaken as part of the annual shut-down, were "ambiguous", says DoE's radiochemical inspectorate, and the procedure for communications between managers was "inadequate, prone to error and not sufficiently formalized". Records were kept only in manual log books and instructions were confused by contradictory suggestions made in pencil.

As soon as the mistake was detected the flow was stopped. Attempts were then made to return the solvent and crud to the treatment plant, but because the available pipeline had only a 2-inch diameter, the procedure was partly unsuccessful and the level of gamma radioactivity near the sea tank outlets necessitated evacuation of workers from the immediate vicinity of the pipes. By this time, senior management had been alerted and after further unsuccessful attempts to rescue the position they decided to flush the offending material down the sea pipeline to increase flow rates and reduce radiation dose to workers. DoE says this decision was taken "without adequate assessment of the available options": the presence of the solvent and highly active crud "does not appear to have been considered".

The company did not at this stage inform the relevant government departments because the total amount of radioactivity in the sea tank was less than the total authorized discharge; DoE says the company "would have been well advised to do so". BNFL maintains that a high proportion of the 4,500 curies of beta activity in the sea tank was returned to the plant, but NII is sceptical and thinks "a significant proportion" went to sea.

The first indication of problems outside the site was on 14 November, when divers from a ship belonging to the environmental group Greenpeace were contaminated with ruthenium 106 in solvent. Two days later, a DoE inspector was informed that ruthenium had been released but was told neither of the complex circumstances nor that more than 500 curies was likely to have been released. On 18 November, BNFL

noticed a slick of solvent at sea and attempted to disperse it, but subsequently lost the slick. The slick and associated debris were later washed up on the beach, but, according to DoE, BNFL had no effective procedure for recording and assessing results. On 20 November, BNFL advised the public that all was back to normal and that beaches could be used again. This was later contradicted by DoE, and BNFL's decision is now criticized.

DoE says that current authorizations are not adequate to deal with sudden releases of solvent and crud. Although the total amount of radioactivity released was well within authorized limits, the sticky nature of the solvent, together with the highly active crud, led to the formation of local "hot spots" of activity, mainly on flotsam, and these were the main potential hazard.

Although little damage to public health is likely to have resulted from the November incidents, there is more concern over plutonium that is routinely discharged and which, contrary to expectations when the discharge pipeline was built, appears to bind to sediments on the sea bed. DoE's radiochemical inspectorate considers that solvent discharges in the past may be responsible for the enrichment of plutonium in the sea surface and its subsequent transfer to land. The National Radiological Protection Board is investigating whether plutonium in household dust in the Sellafield area could account for a local excess of childhood leukaemias claimed in some recent studies.

NII has required BNFL to make a number of immediate changes to operating procedures, and further long-term improvements are planned. Automatic cut-offs have already been installed to prevent solvent or crud finding its way to the sea discharge tanks.

Both reports are now with the Director of Public Prosecutions. The DoE report finds possible breaches of statutory operating requirements relating to record-keeping and maintaining doses to the public that are as low as reasonably achievable.

Tim Beardsley

New man at zoo

BARRING the unlikely emergence and the even less likely victory of an alternative candidate, Sir William Henderson FRS will succeed Lord Zuckerman as president of the Zoological Society of London. Sir William, a former secretary of the Agricultural Research Council, sees his most important task as reducing the society's budget deficit, now running at £2 million a year. The society has long been plagued with financial problems and many

Seabed geology

Gloria goes west

IN a few weeks' time, the MV *Farnella* will sail for the west coast of the United States carrying the unique long-range sidescan sonar "Gloria". The voyage is the result of a new cooperative agreement between the Institute of Oceanographic Sciences (IOS), a component body of the UK Natural Environment Research Council, and the US Geological Survey. It will cost the latter over £1 million.

Gloria (Geological Long Range Inclined Asdic) will be used to survey the ocean floor in all waters more than 500 m deep within the Pacific margin of the recently created US Exclusive Economic Zone. The objective is to produce maps of the shape and texture of the seabed, the interpretation of which may reveal features of economic as well as academic interest.

Gloria was developed by IOS and is at present the only sonar of its type in the world. Using acoustic back-scattering in a manner analogous to the use of oblique illumination during aerial photography, the system produces images of the seafloor up to 30 km either side of the track of the 8-m long towfish. In its six years of service, Gloria II has mapped approximately one-fifth of the world ocean floor.

Geological interpretation of the Gloria data may provide clues to the petroleum potential of the Exclusive Economic Zone. Of equal interest is the possibility of discovering significant quantities of heavy metal sulphides. Hundreds of kilometres of the Gorda and Juan de Fuca ridges lie within the zone and the heat generated at these spreading centres causes large-scale circulation of seawater. This extracts elements such as manganese, zinc, copper, cadmium and silver from the fresh basalt and emerges on the seafloor as spectacular "black smokers". Contact with the cold ocean water causes precipitation of sulphide minerals which may build up economically attractive deposits.

The investigation will yield immediate returns for the academic community but in commercial terms it must be seen as a speculative investment. Peter Gambles

zoo buildings need renovating. Last year, the Department of the Environment turned down an ambitious expansion plan but agreed to write off the society's losses to the tune of £2 million a year until 1986.

Sir William is keen to preserve and enhance the society's scientific prestige: its expertise in reproductive physiology, for example, has allowed it to maintain in captivity species such as the oryx that are almost extinct in the wild. The only way to continue to finance such work will be to increase the number of visitors to the zoos.

Tim Beardsley

Unesco

Turmoil and strife abound

Paris

THE science and technology programmes of the United Nations Educational, Scientific and Cultural Organization (Unesco) are putting a brave face on the threatened withdrawal of the United States at the end of the year. Indeed, there is even a possibility that for the next few months, science and technology, which have often been pleaded in aid in the past year, will be more generously dealt with.

One sign of this is that the annual grant to the International Council of Scientific Unions has been increased by a quarter to \$500,000 for the year ahead, exclusive of contracts yet to be agreed. But Unesco's generosity will also take in for the first time the World Federation of Scientific Workers, which is to receive \$5,000 or more to support its newsletter.

How the total funds for science and technology will be affected will be known only when the urgent review of the budget now under way is decided, in early April. The management has asked for a 10 per cent reduction, but this will not be equally spread. Much the same will happen to vacancies as they arise. Posts will be left vacant if possible but otherwise filled by transfers within Unesco.

Much change is in prospect. The much respected head of the Man and Biosphere Programme left earlier this month for the University of Montpellier. The Belgian head of the Science Policy Division, the work of which was criticized at the biennial General Assembly last year (particularly by the British), will retire later this year. It will be a test of Unesco's managers' mood to see whether that vacant post is filled. One other senior departure seems in the offing at least, but there is talk of a senior Soviet scientist joining.

The mood among the staff ranges from optimism to despair. Some see the director-general's attitude as intransigent, fear their life's work is being jeopardized — "It's just as much my Unesco as his" — and fear that prospects of promotion are blocked. Others consider that less money may encourage efficiency and point out that their financial contribution to outside activities such as training is often only token and could be further reduced without catastrophe.

Some administrative problems are as yet unsolved. For many collaborative projects, the United States provides not only its general contribution but also assistance in kind — research vessels, communications and administration. So will US agencies be allowed (or wish) to continue if withdrawal goes ahead?

The charge of bureaucracy levelled at Unesco is impossible to verify or deny. Some say it is a mess, others point out that repeated enquiries from member governments make endless work, as do the endless

committee meetings at which a Unesco presence is thought necessary. More worrying are the sudden if small shifts of policy at the behest of top management or if an itinerant official should rashly promise to help a host government.

US medical schools

Admissions coaching challenged

Washington

THE administrators of the standard test for admission to US medical schools are taking new legal action to prevent a Pennsylvania company from using bootlegged copies of past tests. The Association of American Medical Colleges (AAMC), which owns the standard Medical College Admission Test, or MCAT, charged in federal court last week that Multiprep, a company that offers to prepare students for MCAT, had reproduced questions from the tests verbatim in its course materials in violation of copyright law.

Multiprep is one of many small organizations offering coaching courses for students planning to take college admissions tests, such as MCAT, that tend to weigh heavily in admissions decisions. The test administrators' standard line has been to discount the effectiveness of such courses, which typically cost several hundred dollars, but otherwise to view them as a tolerable nuisance.

Last June, however, AAMC noticed that 250 students from the Philadelphia area who took MCAT in April 1983 did particularly well on questions that had been recycled from the test given in autumn 1980. Further investigation revealed that all had taken the Multiprep course. AAMC obtained a court order to have Multiprep's offices raided; on the basis of materials seized by federal marshals in the raid, including exact copies of MCAT question papers, AAMC was then granted a temporary injunction against Multiprep pending a trial on the merits of the copyright violation charge. The injunction specifically barred Multiprep from using MCAT materials or from advertising, as it had, its possession of "facsimile" MCAT tests.

In last week's action, AAMC claimed that Multiprep had violated the court order on both counts. AAMC said that 77 of the 87 chemistry and biology questions appearing in Multiprep's current course materials were word-for-word copies of MCAT questions.

According to AAMC, questions are "often" repeated from year to year in the test both for statistical reasons and because of the limited number of "quality" questions covering first-year college material in biology, chemistry and physics. The test administrators take pains to prevent the circulation of test questions;

One impression is that Unesco's most serious problem in science and technology is that it expects its staff to behave as scientists when they are administrators, that the organization is as a result too ready to make judgements off its own bat without the benefit of external advice, and that the endless search for programmes that sound well often blinds it to projects it could usefully tackle.

John Maddox

question papers are numbered serially and are collected and checked after each administration of the test. AAMC officials believe that Multiprep obtained their material by having an examinee remove the inner pages of a test booklet, replacing them with dummy pages.

No date has been set for the trial on AAMC's original accusation of copyright infringement, in which AAMC is seeking \$1.5 million in damages against Multiprep.

Stephen Budiansky

Accident casualties

ROMANIA'S Minister of the Chemical Industry, Gheorghe Caranfil, and his deputy, Ion Bivoralu, were sacked last week as a result of a major accident last December in the pyrolysis installation at the Teleajen petrochemical combine. A joint party and government commission found that the accident was due to "serious deficiencies" in the organization and management of the pyrolysis installation during its commissioning, violations of safety regulations and "serious violations" of order and discipline. The Communist Party Secretariat of Prahova County (in which the Teleajen plant is situated) was rebuked for failing to take "appropriate political educational measures" to ensure the smooth operation of the plant, and the first secretary of the county party committee, Virgil Cazacu, and also a deputy prime minister, Ion Nicolae, received votes of censure.

No details of the accident have been published, but the tone of the official announcements, with their repeated use of the words "grave" and "serious", as well as the penalties imposed, point to major human and financial loss.

The petrochemical industry is a particularly sensitive issue in Romania. Mrs Elena Ceaucescu, the wife of the party leader, is herself a petrochemical engineer, and, as head of the National Council for Science and Technology, was responsible for the expansion of Romania's petrochemical capacity in the early 1970s — a programme which caused Romania, an oil-producer, to become a net oil importer, and which has been a major cause of the country's present energy problems.

Vera Rich

Palaeontology

New bones for old

AFTER 12 years of planning and legal battles, one of the world's top ten palaeontological sites — at Messel, near Darmstadt in West Germany — has been designated a rubbish dump. The decision, which will not be immediately disastrous, is a compromise between the obligation of the local bureau of mines to deal with dangerous old mine workings and the scientific community represented principally by the Senckenberg Museum in Frankfurt.

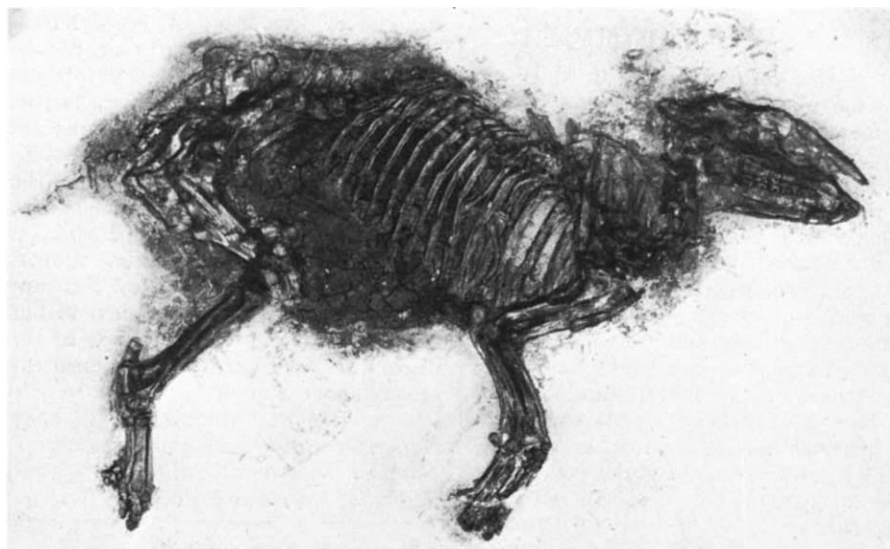
The main site covers about 10 per cent of a 90-year-old opencast mine working in soft oil shale that forms a pit 1 km long and 60m deep. An entire Eocene ecosystem is preserved with a huge variety of fossils of outstanding quality.

The first fossil, a crocodile, was found at Messel in 1875. Between 1886 and 1971, some 20 million tonnes of oil shale were mined. As the mining decreased in the 1960s, the pit became the focus of a rush of amateur fossil hunters. It was eventually closed to the public because of its dangerous state but, with a five-year grant from the Volkswagen Foundation, scientific excavation began in 1976.

To preserve the complete site permanently for palaeontologists would cost DM30 million (£7.5 million) on one estimate, a sum that could not be found by the mining authority of the *Land* of Hesse which has legal responsibility for the site. It has therefore decided that the pit will be filled with waste, although about 40 per cent of the area will be preserved for 20 years for scientific work, and an extra 9 hectares will be made accessible for a period of a year or two while drainage is carried out.

The site is the product of two rivers which bore detritus into a warm (20–30°C) still lake over a period of 1–2 million years. The oil shales preserve not only the contents of the lake but also the flora and fauna of the teeming palm forests that lined its margin. The fossils contain 40 per cent water and were difficult to preserve until the advent of replacement techniques using water-miscible plastics. These reveal skeletons and unique soft-part detail, fine enough to show replacement of many structural parts by bacteria and including feathers and hair. Stomach contents show, for instance, that the bat *Palaeochiropteryx tupaiodon* lived primarily on Lepidoptera and that the horse-like *Propalaeotherium* ate foliage including bay leaves and vines.

Every major group of the animal kingdom is represented at Messel and published papers already fill more than 130 monographs issued by the Senckenberg Museum. Finds have included many rarely preserved amphibians, skeletons of bats with hair and skin, a unique rodent genus



Propalaeotherium messelense, a European relative of the ancestor of the horse.

and, last year, an anteater never before unearthed in Europe and indicative of later land links with America than has hitherto been supposed.

Given the legal position whereby responsibility is a matter solely for *Länder* government, even though most of the research on the site is supported by federal agencies, those involved have reluctantly

accepted what appears to be the best compromise. The argument that Messel is so important that it should be designated by the United Nations Educational, Scientific and Cultural Organization as a site of special historical interest neglects the legal fact that an initiative in that direction would have to come from the *Land* of Hesse. □

Israeli Arabs

Finding technical jobs

Rehovot

FEW Israeli scientists can claim to have declined the offer of a job overseas in favour of one at one-quarter of the salary at home; even fewer are Arabs. One who fits both bills is Dr Nicola Yanaki, a nuclear physicist whose decision to work for one of Israel's most successful high-technology companies was made, in part, to help fellow Israeli Arabs.

Yanaki, who lives in the predominantly Jewish town of Upper Nazareth, is product manager for mammography at the Elscint plant in that town and is largely responsible for the company's advanced systems for the detection of breast cancer. Called Man 2 and Man 3, they provide extremely fine resolution and optimal contrast for accurate differential diagnosis.

Yanaki grew up in the town of Ramle, near Tel Aviv, and received his first scientific training at the Arab Orthodox College (a high school) in Haifa. He then went to Tel Aviv University, where, in August 1981, he became the first Israeli Arab to win a doctorate in nuclear physics. During his stay at Tel Aviv University, Yanaki developed and patented a special gamma-radiation detector. This device, while not yet produced commercially, aroused interest among oil companies because it has proved useful in oil exploration projects.

Indeed, it was because of this detector that a US oil company offered Yanaki a well-paid job, which he turned down when

asked to join the staff of Elscint, which now sells \$110 million worth of medical imaging equipment a year. Yanaki clearly feels that he should repay the nation for having provided him with a good education, as well as being motivated by a desire to help other Israeli Arabs. Those with advanced training in science and engineering have limited employment opportunities because many science and engineering jobs are in defence industries and hence, Yanaki says, "are naturally closed to Arabs in present circumstances".

Until these circumstances change, Israeli Arabs with Yanaki's background must look to civilian science-based industry. By taking a job with Elscint, Yanaki feels that he has "opened the way for others". At Elscint itself, this is clearly the case. There are two Arab engineers in Yanaki's own staff, and another Arab, Anvan Nujam, is carrying out research in Elscint's ultrasound division.

At a time when many others are pessimistic about the chance of peace in the Middle East, Yanaki is optimistic, pointing out that the people of the region have everything to gain by working together. He looks forward to the day when he and his colleagues at Elscint will be able to work on joint projects with researchers in neighbouring Arab countries. And, he adds, "I'm sure that day will come more quickly than anyone now believes possible".

Nechemia Meyers

Human experiment

SIR — The relative rarity with which the results of experimental work in human beings are reported in *Nature* must be the excuse, if not a good one, for the publication of a study on pain in which (1) no indication of whether consent was sought is given (and the study design probably excludes the possibility); and (2) pain experienced by individuals was increased by the experimenters' hidden actions. ("Placebo and naloxone can alter post-surgical pain by separate mechanisms", by R.H. Gracely *et al.*, *Nature* 306, 264-265; 1983). Although dental pain may be regarded as trivial, I, and I am sure many of my colleagues, would regard this study as unethical by any standard.

H.A.F. DUDLEY

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● R.H. GRACEY REPLIES — Professor Dudley's letter challenges whether consent was obtained and the ethics of giving a drug that may increase pain. It is unfortunate that the consent procedure was not described in the text. Human research protocols at the National Institutes of Health (NIH) must be approved by a formal review process that includes written informed consent. In this case, the consent form indicated that "withdrawal from the study may be accomplished at any time without jeopardy or prejudice" and informed the subjects that they may receive "a maximum of 10 mg naloxone — a drug that may be less effective than placebo in pain relief and that theoretically may increase any pain". Professor Dudley is correct in citing this omission from the manuscript but he inappropriately suggests that this study could be performed without consent.

His description of this study as unethical is a more serious matter. He criticizes not only our ethics, but those of other investigators. A dose of 10 mg naloxone has been administered to dental patients after third molar extractions in several highly publicized studies, including one published in *Nature* (272, 826-827; 1978). The increase in pain after naloxone administration was slight and caused no excessive discomfort in these experiments. Our use of naloxone was accompanied by knowledge of its effects in previous studies and by an informed consent procedure that included termination at any time. Patients not only completed this study (involving extraction of third molars on one side) but returned for a similar extraction of their contralateral third molars. They described the naloxone experiment as uncomfortable but tolerable.

Professor Dudley is apparently unaware of the previous studies assessing the effects of naloxone on postoperative dental pain or of the magnitude of the naloxone effect. Naloxone causes a minimal and temporary

increase in discomfort in this situation. However, a great majority of human medical research involves discomfort and risks. It is not the presence of these factors but their magnitude, along with important issues of consent and voluntary termination, that are of ethical concern. The discomfort and risk in this experiment were considerably less than those experienced in many routine medical studies. Unfortunately, Professor Dudley has not addressed previous studies (cited in our article) or the relative magnitude of the discomfort, and has also questioned the research policies at NIH.

RICHARD H. GRACEY

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Placebo defect

SIR — A recent light-hearted account of the banning of urea foam insulation in Canada (*Medical Post*, 15 November 1983), has drawn attention to the lack of a word in the English language to describe the opposite of a placebo. A placebo may be defined as something which, in itself, is inactive, such as a simple lactose pill. However, a placebo has been found to be effective therapeutically in about 30 per cent of patients suffering from a wide spectrum of diseases.

Little attention has been paid to the possibility that a harmless substance produces harmful effects when the patient believes it to be toxic. The author of the article in *Medical Post* suggests the word "garblow". The urea foam insulation was banned precipitately by the Government of Canada after a media crusade against the "dangerous" formaldehyde gas which the insulation may emit. Of about 80,000 households which had installed foam insulation, about 10 per cent developed symptoms which they were encouraged to attribute to the formaldehyde vapour. The English have learned to live with the particular peril of foam insulation but there must be many examples of garblow in our own press threatening our health and sanity.

M. J. BARRIE

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Not by corn alone

SIR — With reference to J. R. S. Fincham's interesting article "Transposable elements and plant gene structure" (*Nature* 306, 425, 1983), I write to point out that P.A. Peterson is a member of the faculty of Iowa State University and not, as the article states, of our sister institution, the University of Iowa.

Incidentally, D.S. Robertson, mentioned in the article as the discoverer of the *Mu-1* transposable element, is also a member of our faculty.

ETHAN HACK

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Homer's wine

SIR — Wright and Cattley's chemical hypothesis about Homer's "wine-dark sea" (*Nature* 303, 568; 1983) is both interesting and evidence of a commendable piece of interdisciplinary cooperation.

But the topic of Homer's epithet is many-sided. Any "solution" of the "puzzle of the wine-dark sea" will be a function of one's particular couching of the question. The chemical hypothesis would certainly be appropriate to the question "Why did Homer call the (Mediterranean) sea wine-coloured (he mentions only red wine in his epics) when it is celebrated for its blueness?" The hypothesis that adding alkaline water to the wine of Homer's time and region turned it from red to blue complies with the scientific requirement of being empirically verifiable, in principle if no longer in practice. Yet a positive test result would not solve, or even invariably be appropriate to, other aspects of the puzzle unless one asserted reductionistically that a scientific answer is the "one proper" one.

Among the many other equally tantalizing aspects of the sea/wine topic are:

(1) Irrespective of the actual colour of the wine, should not the phrase "wine-dark sea" be seen purely or primarily as a formulaic device of the oral Homeric tradition, a recurrent epithet or a fixed metaphor? Homer uses it many times in diverse contexts in both the *Iliad* and the *Odyssey*.

(2) Should we doubt that the Mediterranean sea can look wine-red? The classicist Stanford and others have noted that it is at times a "deep purple crimson" or "mulberry" in places. So "wine-dark" could in fact mean "wine-red".

(3) How adequate, in aesthetic and/or other respects, are the various translations of Homer's original Greek phrase, namely οἶνονα ποντον, to quote one inflexion? Down the ages it has been translated into several languages and with divergent meanings. Examples are "wine-dark sea" (Rieux, Shewring), *les vagues vineuses* (Bérard), *weinrote Meer* (Schadewalt), and *wijnkleurige zee* (Boutens). Among English translations, we have variations such as "wine-coloured sea" (Simcox), "wine-faced sea" (Cook) and even "the gloomy flood" (Cowper). The translator Cook, in fact, sees no colour connotation whatever in the original Greek phrase.

(4) If we focus on just one English translation, say "wine-dark sea", and consider it simply as an aesthetically piquant English phrase, then what is its poetic merit as such? This question entails the usual multiplicity of considerations of imagery, associations, allusions, euphonics, and phonetic symbolism, and decisions made in terms of facets of these criteria may scarcely be a function of what colour the wine was.

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What happens in the mantle?

While it is generally agreed that continental drift occurs by the mechanisms of plate tectonics, the forces that drive these motions remain largely undescribed. But change may be on the way.

AFTER more than 20 years of contentment bordering on complacency, the theory of plate tectonics seems in for a bout of disputation that may be as fierce (and as entertaining) as that of the late 1950s, when geophysics was divided into the two camps of neo-Wegenerians and the conservative rest. That the continents move relative to each other is not now substantially in dispute, of course: the reality of that phenomenon, originally inferred from observations of the deep oceanic crust, is now within the range of direct measurement using radio interferometry. The contentious question now is what drives continental drift? And what, in any case, goes on in the mantle on which the tectonic plates appear to float?

One small sign of which way the wind is blowing can be seen in the note by D.P. McKenzie on page 616. Without saying as much, McKenzie deals with a point often raised by his Cambridge colleague, R.A. Lyttleton, in the past few years — how can the new orthodoxy account for the several recognized episodes of mountain-building in regions far distant from the places at which continents are colliding with each other, or where one plate is riding over another, as in the formation of the Himalayas and the Sierras?

On the standard view, the continents, with an average density of 2.8 g cm^{-3} , float on the more dense mantle beneath in the particular sense to which geophysicists have made us familiar — adjustments take place on a timescale comparable with that of the plastic deformation of the solid material of the mantle. What McKenzie now says is that the mantle material beneath continents may differentiate into dense and less-dense components just as it does beneath the Earth's great spreading oceanic crust. But beneath the continents, the lighter material is either added to the basement or intruded at high levels as horizontal basalt slabs. In either case, the result is to make blocks of continental material more buoyant than they would otherwise have been. The result, the numbers suggest, may be some kilometres of uplift.

Orthodox geologists should be content. Mountain-building other than that provoked by the more spectacular tectonic events is made explicable. So too is the occurrence near the surface of metamorphic rocks, some even derived from sedimentary material, which have obviously in their time been deeply buried. This was precisely the phenomenon that

C.J. Allègre and his colleagues from the Institut de Physique du Globe in Paris were describing last month (*Nature* 307, 17; 1984). Whether this simple notion will settle the long-standing controversy about the origin of the Colorado plateau is less certain, given the great pleasure it gives to orthodox geologists.

Meanwhile, it may be thought puzzling that such a simple idea as McKenzie's has not been put forward long before this. The explanation seems to be that geophysicists have been unduly impressed with the apparent constancy of the thickness of the continental crust, almost everywhere within 5 km or so of 35 km, and at the same time attracted by the simplicity of the notion that the continental crust is essentially primitive material which, once formed, remains on or near the surface. That some of it must do so is shown by the ages of the rocks of the ancient shield areas, exceeding 3,000 million years. But not everything is quite that simple.

Dr D.L. Anderson from the California Institute of Technology seems ready to make a more striking break with the new orthodoxy of plate tectonics (*Science* 223, 347; 1984). Starting from his own recent analysis of the speeds of seismic waves in the upper reaches of the Earth's mantle, Anderson outlines a framework within which the entire standard model of what happens in the mantle might be recast. The observations show that seismic speeds vary from one place to another, as gravity anomalies are known to do, while there is the now long-standing expectation that the regions of continental crust will act as an insulating blanket for the mantle beneath, where the temperature should as a consequence be greater than that elsewhere. Briefly, low seismic velocities signal relatively high temperatures.

Part of Anderson's argument is that these lateral temperature gradients must have an important influence on what happens within the mantle. Few will dissent from that. So what happens to the standard view of what happens in the mantle and of what drives continental drift — the notion that the mantle is one huge pattern of thermal convection cells in which hot material rises (along the lines marked out by the spreading centre) and sinks as cold material where the edges of oceanic plates re-enter the Earth by the process called subduction at the continental margins (south-east Pacific) and at island arcs (western Pacific)?

The remarkable feature of this convection process is that it has been so vague. If the present disposition of the continents and of the plates on which they are mounted is simply to be explained by a pattern of convection cells, at least some features of that pattern must have persisted for some 180 million years, the age of the oldest rocks so far found in the oceanic crust (in the south-east Pacific). So one would expect that the outlines of these convection cells would by now have been fully described, and the outlines of their ascending and descending walls drawn out on maps as befits important influences on the surface structure of the Earth. Then, by comparison with some theory of convection, it should be possible to tell what fraction of the driving force is represented by the thermal energy of the Earth's interior and how much by other influences. But in reality, even the depth of the supposed convection cells has been uncertain, although the discontinuity of seismic velocities at 670 km has been most people's favourite candidate.

Anderson's view of the upper reaches of the mantle is radically different. First, he says, it is not even vertically homogeneous but, rather, layered, as would be expected for a large body of material that has undergone continual zone-refining over aeons. The zone-refining consists of the liberation from the deeper rocks of those components which form liquids, or which can melt, and, on the same view, the buoyancy of the molten material is what drives upward convection. The apparent discontinuity of 670 km is not a phase transition within chemically homogeneous material but a region where both chemical composition and physical properties are changing.

If this way of describing what happens in the mantle proves correct, the consequences will be interesting. First, mantle convection need not be a continuous process but may be episodic — indeed, Anderson argues that the fragmentation of Gondwanaland may be most simply understood as the consequence of the accumulation of heat beneath it, setting off the instability that has continued ever since. And — this is the real radicalism — the occurrence of positive gravity anomalies may not be accidental but, instead, a sign that one consequence of mantle activity is that the Earth's moments of inertia can be changed so as to reorient the mantle as a whole with respect to the spin axis of the Earth.

John Maddox

Vision

An old illusion and a new theory of stereoscopic depth perception

from John P. Frisby

OGLE'S induced effect, a visual illusion first studied in the 1930s¹, has over the past year been the subject of an intriguing series of papers²⁻⁵ (the latest of them in this issue of *Nature*, p.632). The reasons for the renewal of interest in the induced effect can be found in the severe challenge it poses for conventional theories of binocular depth perception and the development of a new computational theory of stereoscopic vision within which it can be encompassed.

Stereoscopic depth perception is generally accepted to derive from the slightly different horizontal positions from which the two eyes view the world — small differences arise in the horizontal positions of corresponding points in the left and right retinal images of objects lying at different distances. But Ogle's induced effect is a change in depth perception caused by introducing vertical, not horizontal, disparities between corresponding points in the two images. The effect is usually produced by placing a meridional size lens, which has the property of magnifying an image in one axis only, in front of one eye such that it enlarges the image in the vertical dimension. As this magnification does not affect the horizontal axis, the lens should have no effect on the perception of depth, given conventional theories. In fact, however, depth perception is markedly altered. For example, a fronto-parallel surface appears rotated about the vertical axis through the fixation point, seeming to protrude towards the observer on the right-hand side if the right eye's image is vertically magnified.

Ogle called the illusion the induced effect because it seemed in many ways as if the vertical magnification 'induced' a whole-field magnification of the other image (or its neural representation)¹. This compensated for the vertical size difference introduced by the lens but at the price of creating a horizontal size difference between images which then produced the illusory rotation via the normal mechanisms of stereoscopic vision. Gillam and Lawergren² provide a good review of the attempts of Ogle and others to explain the induced effect.

Recent interest has centred on the relevance of the induced effect to a new computational theory of binocular depth perception advanced by Mayhew and Longuet-Higgins³⁻⁵. The theory was not specifically designed to account for the effect but it predicts its occurrence as an inevitable side effect for any visual system that implements the theory. The fact that the human visual system is susceptible to the induced effect can thus be regarded as evidence that it contains mechanisms

which implement the theory, a claim which has caused some controversy.

The new theory is based on a geometrical analysis of the spatial distribution of horizontal and vertical disparities. Figure A shows a symmetrically converged pair of eyes observing a square lying in the fronto-parallel plane containing the fixation point. Because the sides of the square are at different distances from each eye, each image of the square is a trapezium. Figure B shows how the two images possess horizontal (H) and vertical (V) disparities, defined respectively as the difference in x and y coordinates of matching points. It can readily be appreciated from the figure that the larger the observed square, the larger will be H and V : in other words, H and V will be scaled by retinal eccentricity. It is also true that H and V will vary with the fixation distance (d) and the angle of gaze (g ; see figures C and D) with which the fixation point is viewed because both d and g will affect the relative sizes of images in the two eyes. The equations derived by Mayhew³ that determine the relative contributions of these factors are:

$$V = \frac{xyI}{d} + \frac{ygI}{d}$$

$$H = \frac{x^2I}{d} + \frac{xgI}{d} + \frac{zI}{d^2}$$

where I is the interocular separation and z is the depth relative to the fixation point (both in centimetres; all other quantities in radians). Note that the z term in the equation for H reflects the fact that H values are sensitive to depth differences in the scene, which is why they provide the fundamental information for stereoscopic vision. In sharp contrast, V values have no z term: as a result they can be used to compute the viewing parameters d and g . With these parameters known it is then possible to interpret the ambiguous information in the equation for H and find the value of z : that is, it is possible to extract absolute values for depth differences in the scene being observed. Of course, all these computations depend upon the difficult problem of stereo correspondence having already been solved, that is, the problem of which points in the left image correspond to which points in the right image (for an introduction to this problem see ref. 6, Ch.7).

The equations for V and H are remarkably simple and constitute a theory of binocular depth perception that shows that the absolute depths of all points in a scene can be recovered from visual information alone. Indeed the equations are so simple that, at least in principle, V values for only two matched points (of differing x

eccentricity and not falling too close to the horizontal meridian) are required for the solution of two simultaneous equations in V to recover the parameters d and g . This is remarkable, because it has been a commonplace belief that d and g , so essential for interpreting the horizontal disparities, have to be obtained from extra-retinal information about eye positions. Curiously, although Helmholtz⁷ knew that information about absolute distance was contained in vertical disparities, he gave no details and his comments have been neglected, perhaps because it has been assumed, incorrectly for near distances, that vertical disparities are too small to be detectable by the human visual system.

Why is it that Ogle's induced effect is produced as a side effect of implementation of the theory? It can be seen from figure C that V varies with retinal eccentricity in x , that is, the lines defining the top and bottom of the square become more and more separated as they extend into peripheral vision. Another way of saying this is that the vertical magnification difference between images is scaled by x , and a useful way of visualizing the equation for V is as a plot of the vertical magnification factor (given by V/y) against x . This graph is illustrated in figure E. It is a straight line whose slope is I/d so that, as I is known, the slope directly yields $1/d$. The reason why the slope is inversely proportional to d is that the further away the fixation point, the smaller will be the vertical size difference between images.

The intercept with the ordinate is given by gI/d , which means that it is scaled both by d and by g . For any given d , if convergence is symmetric ($g=0$), then the plot goes through the origin; but if it is asymmetric ($g \neq 0$), then the line is shifted up or down by an amount proportional to g (fig. E). Changes in g do not alter the slope of the line and hence do not have to be taken into account in the computation of d from the gradient of change in vertical magnification with x eccentricity.

Consider now what happens if one image is vertically magnified. This will not alter the slope of the line but it will add a constant to the quantity V/y present at each x eccentricity, thereby reproducing the state of affairs for a non-zero gaze angle. Hence, a vertical magnification of one image will be interpreted as indicating asymmetric convergence. And by some straightforward algebra it can be shown that, if the pattern of H values created by a frontoparallel surface viewed with $g=0$ are found to be present when $g \neq 0$, then they must have arisen from a surface rotated about a vertical axis through the fixation point. Hence any visual system using vertical disparity information for computing the viewing parameters d and g will be susceptible to an induced effect due to a spurious g being computed when vertical magnification is introduced. Interestingly, observers do not report feeling that they are 'not looking straight ahead'

when experiencing the induced effect, so if it is due to a visual computation of g it must be one that is either solely used for interpreting H values or one that is over-ruled by extra-retinal information about the positions of the eyes as far as 'felt position' is concerned.

The logic of Mayhew and Longuet-Higgins's theory of binocular vision is quite independent of the question of whether any particular visual system uses it. That is, the theory provides a method for recovering d and g from visual information alone (for near distances) which may or may not have been discovered during the evolution of biological visual systems. Nevertheless, it is of considerable interest to ask whether the human visual system uses the method and the induced effect is *prima facie* evidence that it does indeed do so. However, the ' d/g hypothesis' of the induced effect, as it has been called⁸, is by no means the only explanation currently being debated (see ref. 2) and it therefore becomes necessary to enquire whether psychophysical details of the induced effect fit its predictions.

To date, psychophysical predictions drawn from the d/g hypothesis have survived attempts to falsify them as long as the requirement is met that experimental conditions present vertical size differences between images which could arise in natural viewing. Not surprisingly, this is a requirement that adherents of the hypothesis regard as reasonable. They point out, for example, that the fact that the induced effect reaches a maximum at magnifications of about 4–6 per cent is as expected because larger magnifications could only be produced by unrealistically large gaze angles.

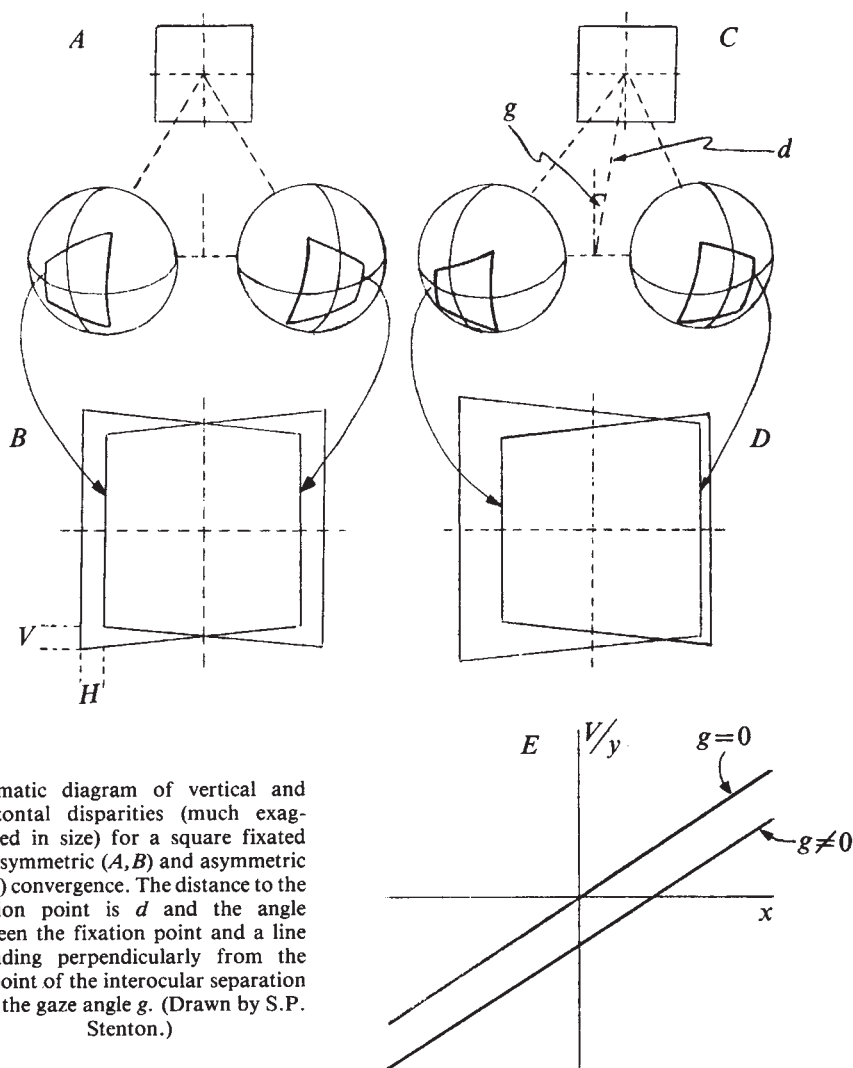
An objection to the hypothesis can be drawn from Westheimer's report⁹ that sensitivity to vertical magnifications falls well below that for horizontal ones. This appears quite at odds with a hypothesis which demands V values be measured with great precision. However, when Westheimer's experimental conditions are examined with the d/g hypothesis in mind, it becomes clear that the large d he used (3 m) means that he was presenting magnifications that would need impossibly large g values to obtain them. In such circumstances, it can be argued that an induced effect would be difficult to observe.

A crucial prediction of the hypothesis is that the size of the induced effect should be scaled by d . Gillam¹⁰, who has independently arrived at a theory of the induced effect which is much the same as that of Mayhew and Longuet-Higgins, recently reported data that she initially thought falsified this prediction. However, when she re-analysed her results excluding those conditions with d/g combinations that could not occur in natural viewing, she found that the remaining data fitted the prediction remarkably well.

One characteristic of the induced effect which Ogle¹ emphasized is that it is very much a 'whole field' phenomenon. For

example, he discovered that an induced rotation can be seen for a field of vertical rods even when only one rod carries vertical disparity information (by fixing some beads to it). That is, V values in one part of the field can create a rotation of the entire field of view. This result is consistent with the d/g hypothesis because d and g are intrinsically whole-field viewing parameters: only one d and g combination can exist at any one time and so it makes obvious sense to compute a single d/g pairing and to use it for interpreting H values over the whole field of view. The same kind of interpretation can be given of results, soon to be reported in *Nature*, showing that it is the average vertical magnification that seems to determine the size of the induced effect⁸. This might reflect a sensible pooling strategy for computing whole-field values of d and g given the small size of vertical disparities.

It may be that the new theory will cause a flurry of new papers on vertical disparity processing as the d/g hypothesis and its competitors are exposed to critical psychophysical examination. If so, this will be a case of experimental interest being led by a clear theoretical exposition of the nature of a given visual computation, and not by the discovery of a new illusion whose potential



computational significance is quite unclear, as has so often happened in the history of psychophysics (for example, selective adaptation effects such as the McCullough effect). Conducting psychophysics within the context of a computational theory is very much in keeping with the research framework proposed by Marr¹¹ and it will be interesting to see whether the outcome in this case will achieve that all too rare occurrence: a clear and definite conclusion about what some psychophysical effects really mean. □

S.P. Stenton is thanked for his help in preparing this article.

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1. Ogle, K.N. *Researches in Binocular Vision* (Saunders, Philadelphia, 1950).
2. Gillam, B. & Lawergren, B. *Percept Psychophys.* **34**, 121 (1983).
3. Mayhew, J.E.W. *Perception* **11**, 387 (1982).
4. Mayhew, J.E.W. & Longuet-Higgins, H.C. *Nature* **297**, 376 (1982).
5. Longuet-Higgins, H.C. *Perception* **11**, 377 (1982).
6. Frisby, J.P. *Seeing* (Oxford University Press, 1979).
7. Helmholtz, H. von *Physiological Optics* Vol.3 (Dover, New York, 1962).
8. Stenton, S.P., Frisby, J.P. & Mayhew J.E.W. *Nature* (in the press).
9. Westheimer, G. *Nature* **307**, 632 (1984).
10. Gillam, B. Paper read to Rank Prize Funds Meeting on *Hyperacuity and Depth Perception* (January 1984).
11. Marr, D. *Vision* (Freeman, San Francisco, 1982).

Cell biology

Membrane traffic and the problem of protein secretion

from Miranda Robertson

THE problem of how to get hydrophilic proteins across hydrophobic membranes is fundamental to both prokaryotic and eukaryotic life; so it would not be unreasonable to expect to find the solution conserved across the evolutionary spectrum from bacterial to animal cells. In fact, however, the conservation of secretory mechanisms is a controversial issue — a state of affairs which may turn out to reflect the existence of more than one secretory mechanism. The present state of the evidence, now beginning to show signs of convergence, was one of the topics of a recent conference* which also dealt with the exclusively eukaryotic problem of what controls the continuous and complex traffic of membrane and secreted proteins through the membrane-bounded compartments of the cell.

The main point of contention is whether secretion in bacteria depends on signalling, guiding, regulating and possibly pore-forming molecules analogous to those that comprise the machinery of transit across the endoplasmic reticulum of eukaryotes¹. It is already well established that most secreted proteins of both bacterial and animal cells are synthesized with amino-terminal leader — or 'signal' — peptides that are enzymatically cleaved from the completed molecule after transport; and that eukaryotic signals are intelligible to bacterial enzymes and vice versa: *Escherichia coli* will, for example, process and secrete insulin² — albeit not very efficiently. Yet according to the work of W. Wickner (UCLA) and L. Randall³, bacteria and even liposomes can transport proteins across their membranes post-translationally and without assistance from proteins other than the leader peptidase^{4,5}; while it is clear that in eukaryotes things are much more complicated.

Two components of the eukaryotic secretory mechanism have already been identified and purified. The first, the signal recognition particle (SRP), binds to the leader peptide of nascent proteins and halts translation until the ribosome has engaged with the endoplasmic reticulum (ER). The SRP is now known to consist of a 7S RNA bound to six proteins⁶, the two smallest of which bind specifically to *Alu* repeats at the ends of the RNA (P. Walter *et al.*, Rockefeller University) — which incidentally suggests that, in general, the hitherto mysterious *Alu* transcripts found in the cytoplasm may all turn out to mediate important functions, and calls into

question the selfishness of the DNA that encodes them.

The second identified secretory component is the docking protein on the cytoplasmic face of the ER. This protein engages with the SRP, releasing the translational block so that synthesis can proceed and the nascent polypeptide is transported co-translationally into the lumen. There is preliminary evidence that the translocation step involves at least one more protein (D. Meyer, EMBL): secreted molecules are not translocated through artificial lipid bilayers supplied only with purified SRP and docking protein. Furthermore, ER membranes inactivated by mild proteolysis can be reconstituted with a purified proteolytic fragment of the docking protein, though after more prolonged proteolysis of the membranes this is no longer possible, presumably because of the destruction of a further component of the translocation mechanism more closely associated with the membrane.

There is clear evidence that the SRP component has been highly conserved in eukaryotic evolution: it can be reconstituted from *Xenopus* or *Drosophila* 7S RNA and mammalian proteins. And now it seems possible that an analogous particle is involved in the secretion of at least some bacterial proteins. Through the analysis of temperature-sensitive mutants of *E. coli* J. Beckwith (Harvard University) and colleagues have been able to identify proteins essential for the secretion of some bacterial products. Antibodies to one of these, the Sec-A protein, precipitates a 6S RNA with some homology to the 7S eukaryotic RNA (H. Liebke, Yale University).

But if Beckwith's work on *E. coli* mutants is beginning to make bacterial secretory mechanisms look more like eukaryotic ones, G. Schatz's (Basel University) analysis of yeast mitochondrial proteins is making eukaryotic mechanisms look more like the prokaryotic ones described by Wickner. According to Wickner, the efficient post-translational transport of the M13 coat protein by *E. coli* is independent of proteins but dependent on membrane potential⁷. The transport of proteins encoded in the eukaryotic nucleus into mitochondria, which can be regarded as an inside-out version of the same process, is also post-translational and dependent on membrane potential. Furthermore, a recent analysis of the effects of mutations introduced into the leader peptides of bacterial secreted proteins lends weight to the increasing evidence³ that there may be not be any fundamental difference between post- and co-translational

mechanisms of transport: M. Inouye (SUNY) reports that certain mutations will convert a co-translationally transported protein into a post-translationally transported one.

The mechanism of transport is not the only important issue that arises in connection with mitochondrial proteins. Unlike the membrane proteins made by a bacterial cell, those made in eukaryotes have a choice of membranes with which to become associated, and must be targeted to the right one. One obvious possibility is that the leader sequences that target proteins to the membrane are specific for different cellular membranes. Not all mitochondrial proteins however are synthesized with leader peptides; and Reizman and his colleagues in Schatz's laboratory have now been able to show that a 70,000-molecular weight protein specifically targeted to the mitochondrial outer membrane in yeast will get lost in the cytoplasm if part of its N terminus is deleted by manipulation of the cloned gene^{7,8}.

The great advantage of yeast for the study of eukaryotic mechanisms is that mutants are relatively easily come by; and it is yeast mutants that may in the end provide the key to one of the most important and least understood of all problems in intracellular traffic: the differential routing of different membrane proteins through the specific formation and fusion of membrane vesicles. It is clear, for example, that once a secreted protein has been successfully extruded into the ER, there is more than one possible route by which it may reach the cell surface; and mutations selectively affecting the different paths would be invaluable. This is the importance of the approach of R. Schekman and his colleagues (University of California, Berkeley) who have isolated temperature-sensitive mutants of yeast blocked at four different stages in the progress of secreted proteins from the ER to the cell surface. The general usefulness of these mutants will of course depend — once again — on how far the secretory mechanisms have been conserved. Schekman and his colleagues have already established, for example, that, as in animal cells, different secreted proteins reach the cell surface at different rates; and that glycosylation is necessary for the correct processing and secretion of some, though not all, of them⁹.

The crucial question, however, is whether these differences are due to the differential interaction of the proteins with components of a common path, or whether they reflect different paths. In animal cells there is little doubt that distinct paths exist — most obviously in those cells in which the secretion of some specialized products is inducible by hormones. Distinctive properties of the inducible path are storage in granules in the absence of stimulation, and the processing of secreted prohormones to their mature form. Thus, for example, if fibroblasts, in which secretion is not normally inducible, are transfected with pro-

*The Ninth EMBO Symposium on Membrane Traffic in the Animal Cell was held in Heidelberg on 26–29 September 1983.

The virus verified

UNTIL the advent of the electron microscope, viruses were to the biologist what atoms had long been to the physicist: entities which could not be seen on account of their small size but which proved exceedingly fruitful in accounting for observed phenomena.

The earliest electron micrographs to reveal unmistakably the head-and-tail structure of viruses were of the viral strain originally called 'anti-coli PC', and now known as bacteriophage T2, taken by Thomas Anderson (RCA Research Laboratories) and Salvador Luria (Columbia University) in the week of 2 March 1942 and published the same year in *Proceedings of the National Academy of Sciences U.S.A.* (28, 127). Anderson recorded the phage particles at a

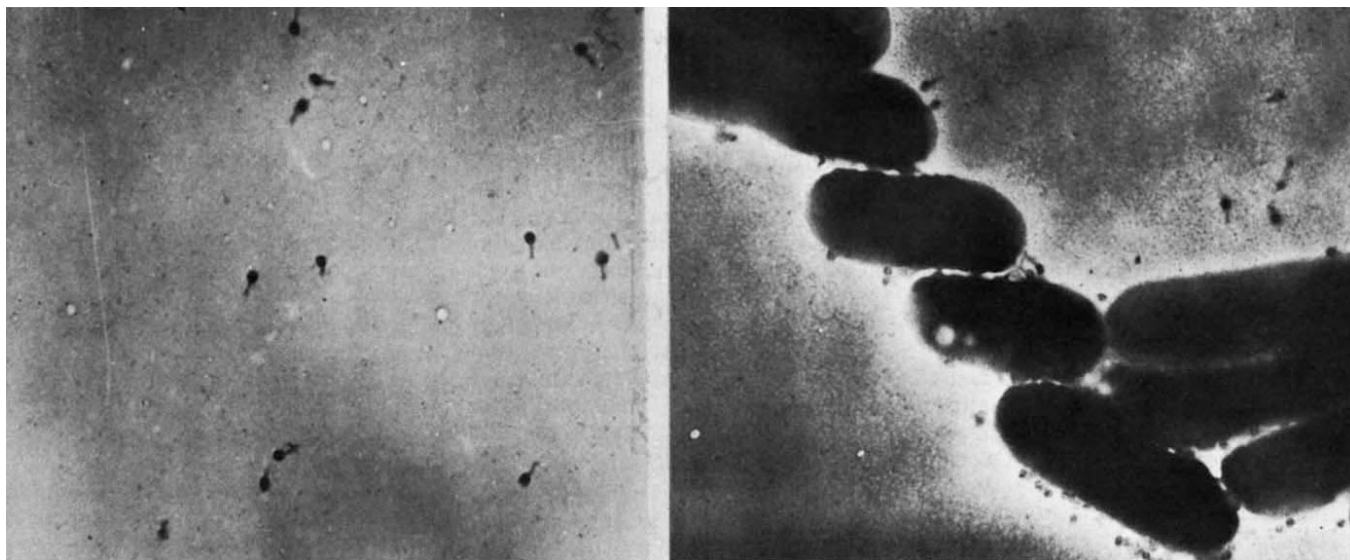
magnification of $\times 45,000$ (below left) and their attachment to the gamma or PC strain of *Escherichia coli* bacilli at $\times 50,000$ (below right), both on Kodak Lantern slide glass plates using an RCA model B electron microscope and very highly concentrated stocks of bacteriophage.

A typical bacteriophage T2 particle possesses a head 60–80 μm in diameter and a tail 120–130 μm long and 20 μm thick. In the right-hand micrograph, the bacteriophage is seen attaching itself to the cell walls of a bacterium, living up to its name of 'bacterium eater'. With hindsight, Anderson describes the ensuing viral attack as follows: rather like a hypodermic syringe, the "attachment causes the tail sheath to contract which drives the needle

[an extension of the neck below the head, invisible on the 1942 micrographs] into the bacterial wall, which somehow induces the DNA contained in the head to pass through the needle into the bacterium. There the DNA takes over the synthetic machinery of the bacterium to make some 100 daughter phage particles in 20 minutes. The bacterium then lyses to release the daughter particles into the medium where each of them can encounter a fresh bacterium to repeat the life cycle'. The infecting phage particles adsorbed onto the bacterial wall remain stuck fast, their delivery complete and their role fulfilled.

Jon Darius

Selected from Beyond Vision by Jon Darius of the Science Museum (Natural History), London (to be published in April by Oxford University Press). Micrographs reproduced by courtesy of T.F. Anderson, now at the Fox Chase Institute for Cancer Research.



insulin they will secrete the unprocessed molecule (R. Kelly, University of California, San Francisco). Conversely, AtT-20 cells, which inducibly process and secrete adrenocorticotrophic hormone, can also be induced to process and secrete insulin after transfection with the proinsulin gene. Unstimulated AtT-20 cells will secrete the unprocessed hormones at low levels, presumably via the constitutive path. This is suggestive, though not strictly conclusive, evidence for two separate vesicular pathways.

And the most recent work of Schekman and his colleagues suggests that yeasts also have distinct secretory paths — although the evidence in this case concerns only constitutive secretion. Schekman and his co-workers find that isolated secretory vesicles contain all the secretory enzymes of yeast but only a subset of the plasma membrane proteins. If the membrane proteins are travelling by different routes, however, they converge at the final step: mutations affecting the last stage in secretion also prevent the appearance of any membrane protein at the cell surface.

For vesicle traffic originating at the cell

surface — and particularly concerning the recycling of membrane receptors — there is some hope that human mutants may soon provide important insights. Receptor recycling has already been reviewed in *Nature*¹⁰ and elsewhere^{11,12} and it is now generally accepted that surface receptors enter the cell via clathrin-coated pits whence they may either return to the cell surface after releasing their ligand, or progress to the lysosomes for degradation.

With the discovery that some forms of familial hypercholesterolaemia are due to mutations that interfere with the biosynthesis or post-translational processing of low-density lipoprotein (LDL) receptors¹¹, it has become clear that defects in cholesterol uptake may make it possible to identify and understand branchpoints in intracellular vesicle pathways. J.L. Goldstein and his colleagues (University of Texas, Dallas) have, for example, identified a mutant receptor that binds cholesterol normally but fails to aggregate in coated pits; and they are now in the process of cloning LDL receptor cDNA, which will enable them to analyse such defects at the molecular level.

Mutant receptors, and mutant viruses borrowing cellular secretory paths, may help to reveal mechanisms that for the time being can only be inferred from morphological and kinetic studies. But the most powerful approach should lie with the temperature-sensitive mutants of yeast: first, because they may affect components of the selective mechanism rather than the selected molecule; and second, because their temperature sensitivity makes it possible to examine defects that would otherwise be lethal. □

Miranda Robertson is an associate editor of *Nature*.

1. see Warren, G. *Nature* 297, 624 (1982).
2. Talmage, K., Brosins, J. & Gilbert, W. *Nature* 294, 176 (1981).
3. Randall, L.L. *Cell* 33, 231 (1983).
4. Wickner, W. *Trends biochem. Sci.* 8, 90 (1983).
5. Ohno-Iwashita, Y. & Wickner, W. *J. biol. Chem.* 258 1895 (1983).
6. Walter, P. & Blobel, G. *Cell* 34, 525 (1983).
7. Reizman, H. *et al. EMBO J.* 2, 216 (1983).
8. Hase, T. *et al. EMBO J.* 2, 169 (1983).
9. Novick, P. & Schekman, R. *J. Cell Biol.* 96, 541 (1983).
10. Hopkins, C.R. *Nature* 304, 684 (1983).
11. Brown, M.S., Anderson, R.G.W. & Goldstein, J.L. *Cell* 32, 663 (1983).
12. Helenius, A., Mellman, I., Wall, D. & Hubbard, A. *Trends biochem. Sci.* 8, 245 (1983).

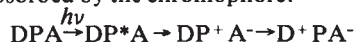
Photochemistry

A quantum step towards artificial photosynthesis

from J. Barber

ACCORDING to an article by Moore and colleagues published in this issue of *Nature* (see p.630), one of the major obstacles to the development of an artificial photosynthetic system has been overcome. For many years the goal of photochemists has been to provide an artificial system that mimics the unique properties of natural photosynthetic reaction centres — particularly their striking ability to facilitate a very rapid and efficient light-induced separation of charge without a significant back-reaction. To date, it has proved difficult to make model systems which do not suffer from high back-reaction rates; now Moore and colleagues seem to have achieved success.

Several different types of reaction centre are found in photosynthetic organisms. Higher plants and algae, which use water as a source of electrons and protons to reduce carbon dioxide and which produce oxygen as a by-product, contain two different types of reaction centre, known as photosystem one and photosystem two. Photosynthetic bacteria, which do not oxidize water, but oxidize more reducing substances such as H_2S and organic acids and therefore do not produce oxygen, contain only one type of reaction centre. Despite these differences, all photosynthetic reaction centres consist of a chromophore (P), which absorbs light energy, and a closely associated electron donor (D) and acceptor (A). The organization of these three components allows the following sequence of events to occur when a photon is absorbed by the chromophore:



The D^+PA^- state is further stabilized by rapid secondary electron transfer processes. P is always a chlorophyll molecule but the chemical nature of D and A varies, with A most often being a quinone. For photosystem one and the bacterial system, D is a metalloprotein, either plastocyanin or cytochrome *c*; the nature of the primary electron donor in the photosystem two reaction centre is unknown but under certain circumstances can be the carotenoid β -carotene. Unlike photosystem one and two, functional reaction centres of photosynthetic bacteria have been isolated free of their light-harvesting proteins and subjected to detailed physical and chemical studies.

With these preparations it has been shown that P^+A^- formation occurs within 100 picoseconds of an excitation flash from an ultrafast laser. The back-reaction is 100 times slower than the forward reaction; normally the P^+A^- radical pair will be

quickly converted to D^+PA^- which then has a much longer lifetime.

Moore and his co-workers have succeeded in synthesizing a very stable molecule which mimics the photochemical properties of natural reaction centres. Their molecule consists of three covalently linked entities: a chromophore (P), a donor (D) and an acceptor (A). The chromophore used is tetraarylporphyrin which has a structure and light-absorption properties comparable to those of chlorophyll. The acceptor is a quinone and the donor is a substituted β -carotene.

Because D and A are spatially separated by the porphyrin, the tripartite molecule has some remarkable properties. Flash spectroscopy shows that D^+PA^- is generated within 200 picoseconds and has a long lifetime — charge recombination occurs on a microsecond time scale. The quantum yield for its formation is also very high and, in certain conditions, can reach 25 per cent. The energy stored in the charge complex is at least 1 V; the conversion of energy is thus remarkably efficient given that only 1.8 eV is available from the absorbed photon. Surprisingly, the

stabilized charge-transfer complex is generated from the singlet state of the excited porphyrin, with triplet states occurring only when the quinone is chemically reduced before illumination. In this situation, the porphyrin triplet is converted in 20 nanoseconds to a less reactive carotenoid triplet by a protective mechanism very reminiscent of a process seen in photosynthetic tissue. In nature, carotenoids not only function to quench chlorophyll triplets but also serve as light-harvesting pigments. Interestingly, Moore and colleagues have found that in their molecular triad, 10 per cent of light energy directly absorbed by the β -carotene moiety is transferred to the porphyrin.

Clearly Moore and colleagues have synthesized a very interesting molecule which, like photosynthetic reaction centres, can efficiently use the singlet energy of an excited porphyrin molecule to bring about stabilization of positive and negative charges to store chemical potential energy. Not only does the molecular triad give a convenient synthetic system for studying very fast photochemical reactions similar to those which occur in photosynthesis, but it could perhaps be exploited in a solar cell in which the molecule would catalyse further secondary reactions which generate a usable chemical or electrical fuel. □

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100 years ago

PROFESSOR Ruskin's utterances are perhaps to be taken least seriously when he is himself most serious, and probably he was never more in earnest than in his jeremiad on modern clouds, delivered at the London Institution on the 4th and 11th inst. Probably none of the readers of *Nature* have been terrified by the storm cloud of the nineteenth century, but should it be otherwise we hasten at once to their relief. Twenty years before the date fixed by Mr. Ruskin for the first appearance of his portentous "plague-cloud", the writer of the present article commenced a series of observations on the forms and structures of clouds, followed a few years later by such daily charts of wind and weather as could be constructed from the data, somewhat meagre, that were then accessible. As might be expected, cyclone and anticyclone were then as they are now. The dimensions and densities of the cloud layers have not altered, neither has our moral degeneracy nor the increased smoke of our manufacturing towns developed any new form of cloud. Neither (until the phenomenal sunrises and sunsets of the last three months) has *Nature*, in painting the clouds, employed upon her palette any fresh tints, whatever artists may have done. Further, we have not observed, nor met with any one, except Mr. Ruskin, who has observed, that the wind during the last thirteen

years has adopted a "hissing" instead of a "wailing" tone.

Admiration ought ungrudgingly to be bestowed on one who has done good service as an art critic and as a contributor to English literature. The sympathy, moreover, which, denied to those who are in advance of their age, is naturally accorded to the archaic type of mind, is enhanced by the attractiveness of a personality whose idealism is as lofty as that of Mr. Ruskin. But we maintain that there is a further sentiment which contributed to the applause which Mr. Ruskin's audiences bestowed upon him. Speaking generally, "broadly and comfortably", as he would say, Mr. Ruskin is not a representative man, yet he represents a certain spirit of Philistinism (for it merits this name), which is far from being unpopular, and which shows itself in opposition to scientific culture. He is the spokesman of that mental attitude which misinterprets the province of science and affects to misunderstand the plainest utterance of the physicist. "The first business", he says, "of scientific men is to tell you things that happen, as, that if you warm water it will boil". "The second and far more important business is to tell you what you had best do under the circumstances — put the kettle on in time for tea." "But if beyond this safe and beneficial business they ever try and explain anything to you, you may be confident of one of two things — either that they know nothing (to speak of) about it, or that they have only seen one side of it, and not only have not seen, but usually have no mind to see, the other".

From *Nature* 29, 353, 14 February 1884.

Palaeoclimatology

Onset of the ice age in the North Atlantic

from Brian Funnell

IN a paper published in this issue of *Nature* (see page 620), Shackleton and colleagues¹ complete a careful interdisciplinary study of sediment cores obtained in the north-east Atlantic and establish with admirable precision that ice-rafted debris first appeared there about 2.5 Myr BP, with the first major input of debris slightly later, at 2.37 Myr BP. The results make it possible to assert with confidence that the North Atlantic and its bordering continents have been subject to periodic glaciation for no less than the past two and a half million years.

Although earlier investigations² of deep-sea sediments had indicated ice-rafting of debris into the North Atlantic from as long ago as approximately 3 Myr, it was not until the hydraulic piston corer was added to the Deep Sea Drilling Project's range of equipment that good-quality cores, suitable for detailed analysis, could be extracted from deep enough into ocean sediments to allow a full interpretation of the time period earlier than half a million years ago.

Shackleton and colleagues investigated one of the earlier cores to be obtained with the hydraulic piston corer (Leg 81, site 552A of the Deep Sea Drilling Project, from a depth of 2,300 metres) using a combination of micropalaeontological, palaeomagnetic and stable oxygen isotope methods.

The magnitude of the oxygen isotope signal (which mainly reflects global ice volumes) and the abundance of ice-rafted debris at 2.37 Myr leave no doubt that the extent of this episode of glaciation in and around the North Atlantic is fully comparable with the most recent glacial episode, which culminated only 18,000 years ago and melted back between 11,000 and 6,000 years ago.

This new date for the onset of general Northern Hemisphere glaciation accords well with a date of 2.4 Myr proposed by workers in the Netherlands³ for the first indication of major cooling in and around the southern margins of the North Sea. Here there is unequivocal evidence of a massive conversion of a temperate forest cover into cold heath conditions, although there is so far no evidence of actual glacierization.

The carbonate content of the deep-sea sediments¹ (which varies in inverse proportion to the ice-rafted component) shows that before 2.5 Myr BP there was no significant sea ice in the North Atlantic. The stable oxygen isotope record shows that before 2.37 Myr ice volume maxima were absolutely less extreme, although subdued

fluctuations, presumably mainly due to variations in the volume of the earlier and older Antarctic polar ice cap, still continue into the more remote past.

From the carbon isotope record of the core sediments it is clear that the formation of North Atlantic deep water by winter cooling at the surface of the Norwegian-Greenland Sea was already established by 3.5 Myr ago, before the onset of glaciation. The surface water, being cold and dense, sinks to the bottom of the North Atlantic. There it gives rise to a higher ¹³C content in bottom-living calcareous microfossils than do the 'older' Antarctic bottom waters

which have had more time to gain ¹²C from the oxidation of ¹²C-rich organic matter falling through the water column.

Since site 552A was drilled, Leg 94 of the Deep Sea Drilling Project has explored six more sites in the North Atlantic using the hydraulic piston corer⁴, with the specific intention of elucidating the history of North Atlantic waters over the last few million years. What we can look forward to now is a detailed spatial and temporal record of past climatic changes in this critical North Atlantic area which will be invaluable for a better understanding of the mechanisms that control its physical oceanography and surrounding climate. □

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1. Shackleton, N.J. *et al. Nature* **307**, 620 (1984).
2. Berggren, W.A. *Init. Rep. DSDP* **12**, 953 (1972).
3. Suc, J.-P. & Zagwijn, W.H. *Boreas* **12**, (1983).
4. Kidd, R.B. *et al. Nature* **30**, 532 (1983).

Neurobiology

Building acetylcholine receptors

from Charles F. Stevens

THE Kyoto group has done it again. Just three months ago, *Nature's* report on the C Cold Spring Harbor Symposium on molecular neurobiology noted that an important advance in channel research could be achieved by the expression in cultured cells of cDNA coding for the four kinds of acetylcholine receptor (AChR) subunits (see *News and Views* **306**, 14; 1983). Numa and his colleagues at Kyoto University have now taken this prize and at the same time made an unexpected observation on the relation between receptor structure and function (see this issue of *Nature*, p.604).

Recall that channels are integral membrane proteins responsible for the electrical activity of the nervous system and that the AChR is the best studied such molecule. The AChR is a pentameric membrane protein composed of four subunits assembled with the stoichiometry $\alpha_2\beta\delta\gamma$. Binding of acetylcholine to the receptor opens the door of a water-filled cation-selective pore for about a millisecond and lets half a million ions traverse the membrane. The channel protein has been purified, partially sequenced, functionally reconstituted, the cDNA for all four subunits has been cloned and sequenced, and genomic clones for at least one of the subunits have been identified and partly sequenced. The function of single copies of the AChR has been studied with the single-channel recording method.

As the report of the molecular neurobiology symposium pointed out, one of the next steps in clarifying the relationship between molecular structure and function would be to get the cDNA expressed in an appropriate system. Once expression has

been achieved, protein structure can be altered rationally and systematically by modifying the DNA, providing a powerful tool for discovering what part subunits, domains and specific amino acids play in the operation of the AChR. The Kyoto group has made a significant start on this programme and has used an interesting approach.

There are three steps to success: constructing an expression vector, finding a method for making appropriate quantities of AChR mRNA and using a system that synthesizes and assembles receptors, and inserts them into surface membrane. Numa and co-workers combined three approaches that previously had been successful in different contexts.

O'Hare *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **78**, 1527; 1981) constructed an expression vector that can be used to transfect cultured cells so that they will express a foreign protein. The Kyoto group used a version of this vector to transfect a cell line, designated COS, prepared by Gluzman (*Cell* **23**, 175; 1981) that contains as part of its genome the simian virus 40 (SV40) DNA required for replication of that virus. The vector used by the Kyoto group contains the replication origin of the SV40 DNA and is thus easily replicated by COS cells: the result is that DNA coding for the AChR subunits is amplified and a relatively large amount of mRNA is made. Cultures were separately transfected with cDNA for each of the four types of AChR subunit, and mRNA coding for each subunit was isolated from the transfected cell lines. This message was then combined and injected into frog oocytes for transla-

tion, receptor assembly and insertion into surface membrane; Barnard *et al.* (*Proc. R. Soc. B* **215**, 241; 1982) had previously used this system to make functional AChRs from message isolated from *Torpedo* electric organs (a rich source of AChR message). The advantages of having separate systems for making message and for using it are that proportions of the message for various subunits can be controlled — for example, mRNA for a given subunit can be completely omitted — and quantity of message can be varied at will.

The Kyoto group find transfected cells make AChR message that is properly processed. Furthermore, when mRNA for all four subunits is injected into oocytes in normal proportions, functional AChRs appear in the surface membrane in numbers that reflect the quantity of message injected; these receptors have apparently ordinary electrophysiological properties and bind appropriately the snake toxin used to identify AChRs. An unexpected result is that functional receptors appeared in oocytes injected with only three of the four subunits. Apparently

one of the larger subunits can substitute for another to form working receptors. Although the possibility of artefact is not entirely ruled out, substitution probably can occur because the gross structure of the various subunits is quite similar — there are strong homologies between all four subunits — and at a certain functional level they are interchangeable. That the two largest subunits are interchangeable supports the pseudosymmetric channel structures that have been proposed by several authors (for references see the paper by Numa *et al.* and the symposium report).

Before this new work from the Kyoto group, the extent to which expression of this rather complicated and delicate membrane protein could be achieved was uncertain. We now know it works; next will come an exploration of altered regulation and function due to systematic and rational changes in amino acid sequence. □

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Plant science

Evolution of the oaks

from Peter D. Moore

OUR knowledge of the fossil history of the oaks has recently been greatly enriched by a description of a range of fossils from an Oligocene locality near Huntsville, Texas, by C.P. Daghljan and W.L. Crepet¹. They have found numerous leaves, fruit and a fragment of a catkin, all of which clearly belong to an oak, or possibly several oak species, and suggest that the oak underwent a rapid evolutionary radiation between the Eocene and the Oligocene.

The genus *Quercus* (the oaks) is a large one, containing somewhere in the region of 300–500 species depending upon whose system of taxonomy you follow. It is characterized within the family Fagaceae (which includes beech and sweet chestnut) by pendulous male catkins, separate female inflorescences and by a nut partially enclosed by a cupule of bracts — the characteristic acorn. Within the genus there is a high level of interspecific fertility, so hybrids are frequent, and there is a great deal of variation in such characters as leaf shape and evergreen or deciduous habit. A high level of variation is also found within many of the species, which further complicates their taxonomy.

A number of attempts have been made to subdivide the genus into subgenera, but authors are not agreed upon the way in which this should be effected. The result is that some species are placed in different subgenera by different authorities. Given such a confused and difficult taxonomic state of affairs among the modern oak, one can only express profound sympathy for

palaeontologists attempting to unravel the fossil history of the genus.

The family Fagaceae was probably present in the late Cretaceous and was certainly well represented by the early Tertiary². *Quercus* wood has been found in Eocene deposits in Britain³ and leaves and fruits in the United States⁴.

The new set of fossils found in Texas were not in intimate association, so it is not possible to be sure whether they do in fact belong to a single species — indeed, careful examination suggests that they possess characters indicative of not only separate species, but even separate sub-genera.

The flower structure within the catkin, with six stamens having extrorse anthers, leads the authors to propose that it belongs to a member of the sub-genus *Erythrobalanus*, currently found in Central and North America. The pollen grains are coarsely scabrate, which is also true of the *Erythrobalanus* oaks. Some leaves found near the flowers resemble those of this sub-genus too, having finely pointed vein endings.

The fossil acorn, however, has basally thickened cup scales which are more frequently associated with another sub-genus, *Lepidobalanus*. This is a rather heterogeneous sub-genus, containing our two British oaks (*Q. robur* and *Q. petraea*), some other European species (such as *Q. ilex*) and some North American species (for example *Q. alba* and *Q. arizonica*).

The outcome of these findings is that the authors propose three new oak species,

assigning the catkin to *Q. oligocenensis*, the leaves to *Q. catahoulaensis* (both sub-genus *Erythrobalanus*) and the acorn to *Q. huntsvillensis* (sub-genus *Lepidobalanus*).

The fact that this mixture of characters should be present in the same sediments, whether or not they are derived from just a single species or several, suggests that *Quercus*, by the Oligocene, had undergone a considerable degree of evolutionary development and had acquired some of the features now characterizing its present-day sub-genera. Before one can accept this rapid evolutionary radiation in oak, however, one must examine the reliability of characters used in defining sub-genera. The subdivision of the genus *Quercus* adopted by *Flora Europaea*, for example, differs considerably from that accepted by Daghljan and Crepet. *Lepidobalanus* is not used there, but some of its members (for example, *Q. robur*) are taken into sub-genus *Quercus* and others (for example *Q. ilex*) to a further sub-genus *Sclerophyllodrys*. Evidently the sub-generic boundaries within the genus are far from clear.

Pollen morphology in *Quercus* has been studied fairly intensively, but the criteria upon which any subdivision of the genus can be firmly based are a matter of dispute. Spoel-Walvins⁵, for example, proposed that some of the south European oak species can be separated by pollen form and sculpturing, but the use of such characteristics in the analysis of Quaternary sub-fossil pollen in Spain has met with very limited success⁶. Examination of type material from numerous sources reveals a very wide degree of intraspecific variations in pollen sculpturing.

One must be cautious, therefore, before fully accepting the rapid evolution of *Quercus* between the Eocene and the Oligocene. The proposal does, on the other hand, tie in well with what is known of environmental conditions in that period. It was a time of climatic cooling, though probably not such a rapid cooling as was once thought⁷, and of falling sea levels that left new tracts of land exposed and created new ecological opportunities. Daghljan and Crepet feel that this environmental instability could underlie the diversification of the genus *Quercus* in the early Tertiary. The next step must be the extraction and analysis of more oak fossils from Eocene and Oligocene rocks in order to examine the extent of this diversification. □

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1. Daghljan, C.P. & Crepet, W.L. *Am. J. Bot.* **70**, 639 (1983).
2. Muller, C.J. *Bot. Rev.* **47**, 1 (1981).
3. Brett, D.W. *Palaeontology* **9**, 86 (1960).
4. Manchester, S.R. *Oregon Geol.* **43**, 75 (1981).
5. Spoel-Walvins, M.R. van der *Acta bot. neerl.* **12**, 525 (1963).
6. Stevenson, A.C. thesis, Univ. London (1981).
7. Collinson, M.E., Fowler, K. & Boulter, M.C. *Nature* **291**, 315 (1981).

Acidic deposition and internal proton sources in acidification of soils and waters

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Enough is now known of the sources of hydrogen ions (protons) in natural ecosystems for the relative importance of external and internal sources to be compared. In acidic soils in the northeastern United States and western Europe, external often exceeds internal proton loading, resulting in soil acidification by aluminium dissolution and sulphate retention. One consequence is the export of ecologically significant quantities of hydrogen ion and aluminium in drainage water.

THERE is considerable controversy over the environmental impact of 'acid rain'. Some researchers claim that atmospheric inputs of anthropogenically-derived acidic substances cause acidification of soils and surface waters with serious effects on vegetation and fish^{1,2}. Others contend that internal proton production in soils, sometimes intensified by changes in land use, overshadow the effects of acidic atmospheric deposition^{3,4}. Thus an evaluation of the relative contributions of atmospheric inputs and internal proton sources to acidification of soil and water would be very useful. We have recently proposed an approach to identify and quantify proton fluxes and soil acidification^{5,6}; we now use this approach to evaluate proton transformations for several ecosystems and discuss the implications for soil acidification and water quality.

Concepts

When evaluating acidification processes it is essential to distinguish between intensity factors (such as pH) and capacity factors (such as acid- and base-neutralizing capacity). Failure to make this distinction⁴ lies at the root of much confusion about the nature of soil and water acidification. In aqueous systems the acid-neutralizing capacity (ANC (aq)) is determined by strong acid titration to a reference pH of about 4.5 and is defined as the aqueous base equivalence minus the strong acid equivalence⁷.

$$\text{ANC (aq; ref. 4.5)} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{A}^-] + [\text{OH}^-] - [\text{H}^+]$$

where $[\text{A}^-]$ is the molar concentration of a potential proton accepting anion (such as a natural organic anion).

In an analogous manner we define the acid-neutralizing capacity of the inorganic fraction of a mineral soil as the sum of basic components minus the strongly acidic components [brackets denote molar concentrations].

$$\begin{aligned} \text{ANC (s)} = & 6[\text{Al}_2\text{O}_3] + 6[\text{Fe}_2\text{O}_3] + 2[\text{FeO}] \\ & + 4[\text{MnO}_2] + 2[\text{MnO}] + 2[\text{CaO}] \\ & + 2[\text{MgO}] + 2[\text{Na}_2\text{O}] + 2[\text{K}_2\text{O}] \\ & - 2[\text{SO}_3] - 2[\text{P}_2\text{O}_5] - [\text{HCl}] \end{aligned}$$

Weak acid components (such as CO_2 (s), SiO_2 (s) and H_2S (s)) do not contribute to ANC (s) because of their inability to accept or donate protons at reference pH values of soils (generally pH 2–5)⁵. Also because the nitrogen content of mineral material is generally low, nitrogen components are not included in our definition of ANC (s).

The capacity factor ANC, rather than the intensity factor pH, is directly influenced by addition or depletion of protons, and hence is fundamental to acidification and alkalization. pH generally, but not always, decreases during addition of protons and generally, but not always, increases during depletion of protons⁵. While pH is of more direct ecological importance than ANC, the relationship between pH and ANC is beyond the scope of this paper. Detailed discussions on the functional relationship between pH and ANC (aq)⁷ or soil mineralogy⁸ (which in turn determines ANC (s)) are available elsewhere.

Soil acidification is defined as a decrease in ANC (s) and is accomplished by removal of cationic components (such as CaO) from a mineral soil or, to a lesser extent, by addition of acidic components (such as SO_3). Conversely soil alkalization is an

Table 1 Proton producing and consuming processes in terrestrial and aquatic ecosystems

Proton-producing processes		Proton-consuming processes
(1) Atmospheric input	$\text{H}^+ (\text{aq}(\text{rain/drain water})) \rightleftharpoons \text{H}^+ (\text{aq}(\text{soil solution}))$	(1) Drainage
(2) Assimilation of cations	$\text{M}^{n+} (\text{aq}) + n\text{R} - \text{OH} (\text{org}) \rightleftharpoons (\text{R} - \text{O})_n \text{M} (\text{org}) + n\text{H}^+ (\text{aq})$	(2) Mineralization of cations
(3) Mineralization of anions	$n\text{H}_2\text{O} + \text{R}_n - \text{A} (\text{org}) \rightleftharpoons n\text{R} - \text{OH} + \text{A}^{n-} (\text{aq}) + n\text{H}^+ (\text{aq})$	(3) Assimilation of anions
(4) Dissociation of acids	$\text{H}_n\text{A} (\text{aq}) \rightleftharpoons \text{A}^{n-} (\text{aq}) + n\text{H}^+ (\text{aq})$	(4) Protonation of anions
(5) Oxidations	$\text{Red} (\text{aq, s, g}) + m\text{O}_2 + n\text{H}_2\text{O} \rightleftharpoons \text{Ox}^{r-} (\text{aq, s, g}) + r\text{H}^+ (\text{aq})$	(5) Reductions
(6) Reverse weathering of cations	$\text{M}^{n+} (\text{aq}) + \frac{1}{2}n\text{H}_2\text{O} \rightleftharpoons \frac{1}{2}n\text{M}_{2/n}\text{O} (\text{s}) + n\text{H}^+ (\text{aq})$	(6) Weathering of metal oxide components
(7) Weathering of anionic components	$\text{NO}_{(m+n)} (\text{s}) + m\text{H}_2\text{O} \rightleftharpoons \text{NO}_{(2m+n)}^{2m-} (\text{aq}) + 2m\text{H}^+ (\text{aq})$	(7) Reverse weathering of anions

Quantitatively important cations (M^{n+}) include Ca^{2+} , Mg^{2+} , Na^+ , K^+ (equations (2), (6)), NH_4^+ (2) and Al^{3+} (6). Important anions (A^{n-}) are H_2PO_4^- (equations (3), (7)), NO_3^- (3) and SO_4^{2-} (3, 7). Mineralization and assimilation of SO_4^{2-} and NO_3^- involves oxidation and reduction processes but the net reactions are equivalent to equations (3). Important reduction (Red)–oxidation (Ox) couples in gas/water/soil systems include $\text{NH}_4^+/\text{NO}_3^-$, N_2/NO_3^- , $\text{H}_2\text{S}/\text{SO}_4^{2-}$, $\text{SO}_2/\text{FO}_4^{2-}$ and $\text{Fe}^{2+}/\text{Fe}_2\text{O}_3$. Reduction/oxidation reactions (5) have been coupled to the O_2 – H_2O system. In actuality, organic matter (CH_2O) is usually the electron donor in reduction reactions (5); CH_2O oxidation is implicitly expressed in this reaction because organic matter is oxidized during aerobic respiration ($\text{O}_2 + \text{CH}_2\text{O} \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$). Cation and anion exchange reactions can be represented by reactions (6) and (7).

Table 2 Proton fluxes, rates of soil acidification, and external/internal proton ratios of different ecosystems

Case	Ecosystem	Soil, vegetation	Measuring period	H ⁺ -sources (kmol hectare ⁻¹ yr ⁻¹)					H ⁺ -sinks (kmol hectare ⁻¹ yr ⁻¹)					External/ internal proton ratio	Δ ANC (s)
				External		Internal									
				Input/N-transfer	CO ₂	Biomass	Weather- ing	Σ+	Σ-	ing	Biomass	N-transfer	Stream		
1	World average	No net biomass assimilation	—	0.0/0.1	2.6	0.0	0.5	3.2	3.2	3.2	0.0	0.0	0.0	0.03	-2.7
Soil alkalization															
2	Lake Chad, Chad	Bottom of alkaline lake	1967-72	0.0/0.0	-3.3*	0.0	3.4	3.4	3.3	0.0	0.0	0.0	0.0	0	+3.4
Low rates of soil acidification															
Bogs															
3	Thoreau's bog, Mass.	Sphagnum bog	1976-77	0.9/0.0	0.5†	0.1	0.0	1.6	1.3	0.4§	0.3	0.1	0.5	1.33	-0.1
4	Storgama, Norway	Oligotrophic peat, pH 4	1974-76	0.5/0.2	0.3‡	0.0	0.0	1.0	0.9	0.4	0.0	0.2	0.3	1.67	-0.4
Well drained soils															
5	Birkeness, Norway	80% Forested podzols, 20% peat	1974-76	1.2/0.7	0.5‡	0.0	0.0	2.4	2.3	1.3	0.0	0.7	0.3	2.40	-1.3
6	Lüneburger Heide, FRG	Haplorthod, Calluna	1978-79	0.6/0.5	0.0	0.6	0.7	2.4	2.4	1.6	0.3	0.2	0.2	0.69	-0.9
7	HBEF, New Hampshire	Haplorthod, pH 3.6-5, hardwoods	1963-74	1.3/0.1	0.1	1.0	0.1	2.6	2.5	2.2	0.2	0.0	0.1	1.17	-2.1
8	Gårdsjön, Sweden	Podzols, pH 4.5, <i>Picea</i> , <i>Pinus</i>	1979-81	1.0/0.7	0.7‡	1.0	0.3	3.7	3.5	2.4	0.0	0.7	0.5	0.50	-2.1
9	Mondo¶, Indonesia	Andosol, pH 5.5, <i>Agathis</i> plantation	1976-77	0.0/0.0	4.0	1.2	1.4	6.5	6.5	3.5	2.8#	0.2	0.0	0	-2.1
Intermediate rates of soil acidification															
10	Solling, FRG	Acid brown forest soil, pH 4, <i>Fagus</i>	1969-79	1.7/0.8	0.0	1.5	0.1	4.1	4.3	3.0	0.3	0.5	0.5	1.25	-2.9
11	Solling, FRG	<i>Picea</i>	1973-79	3.7/1.1	0.0	2.1	0.2	7.1	7.0	6.1	0.6	0.0	0.4	2.09	-5.9
12	Hackfort A, Neth.	Haplaquept, pH 4, <i>Quercus</i> , <i>Betula</i>	1981-82	0.4/4.2	0.0	0.8	1.1	6.4	6.2	6.1	0.1	0.0	0.1	2.42	-5.0
13	Hackfort B, Neth.	Dystrochrept, pH 4, <i>Quercus</i>	1981-82	0.4/3.9	0.0	1.1	0.8	6.2	5.8	5.6	0.1	0.0	0.1	2.26	-4.8
14	Hackfort C, Neth.	Udipsamment, pH 4, <i>Quercus</i> , <i>Betula</i>	1981-82	0.4/2.6	0.0	0.7	0.7	4.4	4.4	3.9	0.1	0.3	0.0	1.93	-3.2
15	Andrews**, Oregon	Dystrochrept, mature <i>Pseudotsuga</i>	1973-75	0.3††/0.0	0.9	0.0	3.2‡‡	4.5	4.4	4.4	0.0	0.0	0.0	0.07	-4.4§§
High rates of soil acidification															
16	North. Cascades, Wash.	Quartz-diorite + glacial till no vegetation	1970	0.4/0.0	7.2	0.0	2.2‡‡	9.8	9.9	9.9	0.0	0.0	0.0	0.04	-9.9§§
17	Hackfort D, Neth.	Haplaquoll, pH 7 <i>Quercus</i> , <i>Alnus</i> , <i>Populus</i>	1981 (1 event)	0.4/5.9	1.7	2.4	0.8	11.1	10.1	10.0	0.1	0.0	0.0	1.07	-9.2
18	Castricum I, Neth.	Undipsamment, 3.5% CaCO ₃ , no vegetation	1952 (5 events)	1.5 /1.0	12.6	0.0	0.0	15.1	15.6	15.6	0.0	0.0	0.0	0.20	-15.6
19	Castricum III, Neth.	Udipsamment, <i>Quercus</i>	1952 (5 events)	1.6 /0.6	12.4	0.0¶	0.2	15.0	15.1	14.5	0.0	0.6	0.0	0.13	-14.3
20	Castricum IV, Neth.	Udipsamment, <i>Pinus</i>	1952 (5 events)	1.7 /0.6	7.8	0.1¶	0.2	10.3	10.3	9.7	0.0	0.6	0.0	0.21	-9.5
Deforested system															
21	HBEF, New Hampshire	Haplorthod, pH 3.6-5; deforested	1966 (2 events)	1.0/11.2# #	0.0	0.8	2.1	15.1	13.9	4.3	5.9	3.1	0.6	0.07	-2.2

See Table 3 for references and hydrological characteristics of ecosystems.

* Proton neutralization due to protonation of HCO₃⁻.

† Organic acid deprotonation rather than CO₂ deprotonation (measured).

‡ Organic acid deprotonation rather than CO₂ deprotonation (estimated by discrepancy in charge balance of drainage water).

§ Largely sulphate reduction.

|| Proton neutralization due to sulphate reduction is not included in Δ ANC (s).

¶ Anion input, output and assimilation data from L. A. Bruynzeel (personal communication). The assumptions are: sulphur input equals sulphur output; HCO₃⁻ in drainage water estimated from charge balance discrepancy.

High rates of net Na⁺, K⁺ and Mg²⁺ mineralization in a young (recently cut, <40 yr) forest plantation.

** Input of Cl⁻ and output of SO₄²⁻ are estimated from discrepancy in charge balance. Data from weir are used as output. Cl⁻ output is assumed to equal Cl⁻ input.

†† Dry deposition of S was assumed to be SO₄²⁻ in throughfall less SO₄²⁻ in precipitation.

‡‡ Assumed to be due to pyrite (FeS₂) oxidation.

§§ The transformation of H₂S₂ (s) to SO₃ (aq) that is removed by leaching does not change ANC (s), so Δ ANC (s) is equivalent to H⁺ consumed in weathering.

||| Wet deposition was estimated from Cl⁻ output in the unvegetated lysimeter, assuming that Cl⁻ input equalled Cl⁻ output and the stoichiometry of ions in precipitation in 1952-55 were similar to values in 1980¹⁴. Sea spray on vegetation surfaces was calculated by assuming that Cl⁻ output less Cl⁻ input equals Cl⁻ dry deposition.

¶¶ Any SO₄²⁻ output that exceeded SO₄²⁻ inputs from wet deposition and sea spray was attributed to dry deposition of SO₂.

¶¶ Biomass accretion was estimated by measured increases in tree height and literature values of nutrient content^{15,16}.

Mineralization of organic N.

increase in ANC (s). Changes in ANC (s) may involve both the weathering/formation of soil minerals, and adsorption/desorption reactions. Although Fe₂O₃ and Al₂O₃ are included in ANC (s), they are relatively insoluble at pH values above 3 and 5 respectively and therefore are important only in the acidification of moderately to strongly acidic soils. Oxidation of FeO to Fe₂O₃, common in weathering zones with FeO-containing minerals, increases ANC (s). Because this increase is difficult to quantify and is of no practical significance we will neglect the effect of Fe₂O₃ on ANC(s).

Soil acidification occurs by an irreversible flux of protons to the soil. Proton sources include: (1) atmospheric inputs of acidic or potentially acidic substances (such as SO₂); (2) net assimilation of cations by vegetation; (3) net mineralization of anions

from organic matter; (4) deprotonation of weak acids; (5) oxidation reactions; (6) precipitation (reverse weathering) of cations; and (7) mineral weathering of anionic components (Table 1).

Protons entering or produced in the soil may be removed by (1) export in drainage water, (2) net mineralization of cations in organic matter, (3) net assimilation of anions by vegetation, (4) protonation of anions, (5) reduction reactions, (6) weathering of cationic components and (7) precipitation of anions (Table 1).

Free or bound protons (such as H₂CO₃) are transferred to the soil predominantly through the dissolution of cationic components.

If a soil completely neutralizes proton inputs, basic cations

(Ca^{2+} , Mg^{2+} , Na^+ , K^+) are released and exported from the soil by vegetation uptake or by outflow of drainage water, resulting in soil acidification. Aqueous alkalization (an increase in ANC (aq)) generally occurs as a counter-process to soil acidification. Alkalization of the percolating soil solution is accomplished by neutralization of free protons in solution by soil, or by the donation of bound protons to the soil resulting in the formation of unprotonated anions (such as HCO_3^-).

If there are considerable proton inputs to a soil, and soil-bound basic cations are present in low concentrations or have characteristically slow dissolution kinetics, or the retention time of water within the mineral soil is short, the neutralization of proton inputs may be incomplete and result in the export of free protons in drainage water from the soil. A consequence of elevated concentrations of free protons (low pH) is the dissolution of aluminium from soil. Aluminium dissolution is realized in acidic conditions ($\text{pH} < 5.5$) and results in a solution that contains both hydrogen ion and aluminium base neutralizing capacity⁹:

$$\text{BNC (aq, ref. pH 5.5)} = \text{H}^+ + n[\text{Al}^{n+}].$$

Elevated levels of dissolved aluminium are ecologically significant due to toxicity to aquatic organisms^{10,11} and vegetation¹².

There are two approaches to the quantification of soil acidification. A historic approach requires the determination of ANC (s) in a complete soil profile, including parent material, or a chronosequence of soils. An actualistic approach involves the measurement of all proton fluxes so that a proton budget can be calculated. The measurements required to compile a proton budget include: the flux of solutes entering and leaving the ecosystem; the net assimilation (or mineralization) of substances by living or dead biomass; the net quantities of potentially acidic gases and aerosols entering and leaving the ecosystem; and the net sorption (for example, by precipitation) or desorption of substances in the soil. The advantages of the proton budget approach over the historic approach are obvious; it reflects the current conditions of an ecosystem, identifies various processes involved in soil acidification, is sensitive compared with the analysis of soil material and requires no assumptions about the authenticity of soil parent material. In our discussion of soil acidification we will follow only the proton budget approach. Further details on the processes of soil acidification and methods of quantifying soil acidification are available elsewhere⁵.

Proton accounting

We have developed proton budgets using data from several ecosystem studies (Table 2). To facilitate our discussion we have categorized total proton sources and sinks, for each ecosystem into their principal constituents. Proton sources include: (1) atmospheric inputs of free protons and SO_2 ; (2) the proton loading associated with nitrogen transformations, which is the net ammonium input plus the net nitrate output (this accounts for the protons generated during the net assimilation of atmospheric NH_4^+ by biota and net nitrification); (3) the deprotonation of CO_2 or organic acids; (4) net assimilation of non-nitrogen cations by biomass (or net non-nitrogen anion mineralization); and (5) anion weathering (plus any reverse cation weathering).

We have subdivided proton sinks into: (1) cationic weathering (plus any reverse anionic weathering); (2) biomass assimilation of non-nitrogen anions; (3) proton neutralization associated with nitrogen transformations (which is net nitrate input/net ammonium output); and (4) drainage export of free protons.

We define external proton inputs (which we presume are largely anthropogenic in origin) as atmospheric inputs of H^+ and SO_2 plus proton sources minus proton sinks due to N transformations or, in other words, net ammonium input minus net nitrate input. The balance of proton sources (3), (4) and (5) will be considered as internal proton loading. It is invariably arbitrary to partition proton loading into internal and external sources. For example, atmospheric NH_4^+ is generally derived

from NH_3 volatilization and protonation by acidic atmospheric substances (for example, H_2SO_4 from SO_2 , HNO_3). Therefore protons generated from assimilation or oxidation of atmospheric NH_4^+ in the soil clearly reflect an external source. On the other hand in degrading ecosystems mineralization of organic nitrogen followed by nitrification will result in net internal generation of protons. However, nearly all ecosystems considered here increase in biomass or are at steady state, with net ammonium inputs generally exceeding net nitrate outputs. Therefore exported nitrate can be generally assumed to come from atmospheric NH_4^+ , so that the proton loading from nitrogen transformations is considered here as an external source. Note that atmospheric N_2 fixation involves no proton transfer. Only after mineralization of organic N to NH_4^+ or NO_3^- will fixed N_2 become involved in proton transfer. This process is accounted for by an input-output budget of NO_3^- and NH_4^+ .

To assess the relative importance of external proton inputs to the total sources of an ecosystem, we define the external-internal proton ratio (EIPR) as the ratio of atmospheric inputs of mineral and potentially mineral acids to the internal proton loading.

The rate of soil acidification is equivalent to the proton flux associated with anionic weathering minus the proton flux associated with cationic weathering. The summed proton sources and sinks should, theoretically, be equal. A comparison of the total proton sources and sinks provides an estimate of the accuracy of the budget. Budget discrepancies may be due to errors in measurements or to proton transfer processes that have been overlooked.

To illustrate the nature of soil acidification and its role in regulating drainage water quality, we have also tabulated the constituents contributing to $\Delta\text{ANC (s)}$, $\Delta\text{ANC (aq)}$ and the transport of BNC from the ecosystem (Table 3). The contributions to $\Delta\text{ANC (s)}$ have been subdivided into changes in basic cation components (CaO , MgO , Na_2O , K_2O), aluminium components (Al_2O_3) and anionic components (SO_3 , P_2O_5 , HCl). Because most soils considered here are oxidized and without extremely low pH values, soil acidification due to changes in iron and manganese component content is generally insignificant. Changes in ANC (aq) have been attributed to changes in free proton, bicarbonate, and organic anion concentration. Because of the ecological significance of aqueous acidity, we have also tabulated the quantities of dissolved hydrogen ion and aluminium exported from soils as well as the pH of the drainage water.

Soil acidification and proton sinks

There is considerable variation in the extent and nature of proton fluxes in ecosystems (Table 2). Data from the global terrestrial ecosystem (case 1) and sites where precipitation inputs exceed evapotranspiration indicate that soils generally undergo acidification (ΔANC is negative), while water percolating through the soil increases in ANC (aq). However, in arid or semi-arid regions, like Lake Chad (case 2), the supply of solutes exceeds leaching of solutes and reverse weathering occurs (such as CaCO_3 , or smectite formation), resulting in soil alkalization.

Although most soils acidify, the extent to which this process occurs is highly variable and essentially depends on soil type, geology and the quantity of drainage water. We have subdivided ecosystems into those with: (1) low rates of soil acidification ($< 2.5 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$); (2) intermediate rates of soil acidification ($2.5\text{--}7.5 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$) and; (3) high rates of soil acidification ($> 7.5 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$).

Ecosystems with low rates of soil acidification often have acidic podzolic soils which are derived from granitic or base poor materials (cases 5–8), strongly weathered volcanic materials (case 9) or are bogs (cases 3, 4). Ecosystems with intermediate rates of soil acidification generally have acidic soils containing appreciable amounts of weatherable silicate minerals. Examples include soils that are derived from young volcanic materials (case 15), decalcified fluvial deposits (cases 12–14) and decalcified loess (cases 10 and 11). Soils with high rates of

Table 3 Constituents contributing to the rate of soil acidification ($\Delta\text{ANC (s)}$) and to the change in acid neutralizing capacity of water percolating through the soil ($\Delta\text{ANC (aq)}$) and the transport of BNC (aq) in drainage water

Case	Ecosystem	Ref.	mm yr ⁻¹		$\Delta\text{ANC (s) (kmol ha}^{-1}\text{ yr}^{-1})$				$\Delta\text{ANC (aq) (kmol ha}^{-1}\text{ yr}^{-1})$				BNC (aq) (kmol ha ⁻¹ yr ⁻¹)			pH of drainage water
			Rainfall	Runoff/percolation	Total	Cations	Anions	Aluminium	Total	HCO ₃ ⁻	A ⁿ⁻	H ⁺	Total	H ⁺	Aluminium*	
1	World Average	17	800†	280†	-2.7	-3.2	+0.5	0.0	2.6	2.6	0.0	0.0	0.0	0.0	0.0	8.3
Soil alkalization																
2	Lake Chad	18	275‡	245	3.4	3.4	0.0	0.0	-3.3	-3.3	0.0	0.0	0.0	0.0	0.0	—
Low rates of soil acidification																
Bogs																
3	Thoreau's bog	19, 20	1,450	240§	-0.1	-0.1	0.0	0.0	0.9	0.0	0.4	0.5	0.5	0.5	0.0	3.7
4	Storgama	21	920	530	-0.4	-0.3	0.0	-0.1	0.4	0.0	0.3	0.1	0.4	0.3	0.1	4.2
Well drained soils																
5	Birkeness	22	1,290	1,030	-1.3	-0.5	-0.1	-0.6	1.4	0.0	0.5	0.9	0.9	0.3	0.6	4.5
6	Lüneburger Heide	23	765	614	-0.9	-1.2	0.7	-0.4	0.4	0.0	0.0	0.4	0.6	0.2	0.4	4.4
7	Hubbard Brook	6, 24	1,320	835	-2.1	-1.8	0.1	-0.4	1.3	0.1	0.0	1.2	0.3	0.1	0.2	4.9
8	Gårdsjön	25	1,130	665	-2.1	-1.5	0.2	-0.6	1.2	0.0	0.7	0.6	1.1	0.5	0.6	4.2
9	Mondo	26	3,580	2,955	-2.1	-1.5	0.0	0.0	4.0	4.0	0.0	0.0	0.0	0.0	0.0	6.8
Intermediate rates of soil acidification																
10	Solling (<i>Fagus</i>)	27	950	705	-2.9	-0.6	-0.5	-1.5	1.2	0.0	0.0	1.2	2.2	0.5	1.7	4.2
11	Solling (<i>Picea</i>)	27	950	480	-5.9	-1.1	-0.2	-4.3	3.3	0.0	0.0	3.3	4.7	0.4	4.3	4.1
12	Hackfort A	28	650	100	-5.0	-3.4	-1.1	-0.4	0.3	0.0	0.0	0.3	0.4	0.0	0.4	4.5
13	Hackfort B	28	650	103	-4.8	-0.7	-1.7	-2.4	0.3	0.0	0.0	0.3	2.5	0.1	2.4	4.2
14	Hackfort C	28	650	149	-3.2	-0.4	-1.6	-1.1	0.3	0.0	0.0	0.3	1.2	0.0	1.1	4.6
15	Andrews	29	2,370	1,545	-3.6	-4.4	0.0	0.0	1.2	0.9	0.0	0.3	0.0	0.0	0.0	6.7
High rates of soil acidification																
16	Northern Cascades	30	3,840	4,090¶	-9.3	-9.9	0.5	0.0	7.6	7.2	0.0	0.4	0.0	0.0	0.0	7.0
17	Hackfort D	28	650	125	-9.2	-7.4	-1.8	0.0	2.1	1.7	0.0	0.4	0.0	0.0	0.0	7.0
18	Castricum I (unvegetated)	31	800	610	-15.6	-15.6	0.0	0.0	14.1	12.6	0.0	1.5	0.0	0.0	0.0	8.0
19	Castricum III (<i>Quercus</i>)	31	800	350	-14.3	-14.3	0.0	0.0	14.0	12.4	0.0	1.6	0.0	0.0	0.0	7.8
20	Castricum IV (<i>Pinus</i>)	31	800	175	-9.5	-9.5	0.0	0.0	9.5	7.8	0.0	1.7	0.0	0.0	0.0	7.8
Deforested system																
21	Hubbard Brook, deforested	32, 33	1,320	1,075	-2.2	-3.8	-0.4	0.8	0.4	0.0	0.1	0.3	2.6	0.6	2.0	4.2

* For most sites the chemical speciation of Al is unknown, so it was assumed to be trivalent. As a result the contribution of Al (in $\text{kmol } \frac{1}{3} \text{ Al}^{3+}$) to BNC is an upper limit.

† Except Antarctica, data from ref. 34.

‡ River input 2,020 mm yr⁻¹, evaporation 2,100 mm yr⁻¹.

§ Net annual storage of water in the bog was 190 mm.

|| High percolation rate associated with a depletion of soil moisture.

¶ Runoff is greater than precipitation inputs due to glacial melting.

acidification are generally calcareous (cases 17–20). An apparent exception to these subdivisions is the Northern Cascades (case 16). Here soils are thin and derived mainly from schists and quartzdiorite, but rates of soil acidification are extremely high ($-9.9 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$). This apparent anomaly may be attributed to glacial activity and the very large amount of water draining the region.

In well drained soils, weathering of cationic components is the principal mechanism of proton neutralization (Table 2). However, bogs (cases 3, 4) are generally rich in organic matter and weathering of minerals is quantitatively unimportant. In the reduced environment of bogs proton removal is predominantly achieved through biomass assimilation of anions (S, N, P), reduction of atmospherically derived sulphate and nitrate, and export of free protons.

Internal proton sources

In ecosystems with relatively high rates of soil acidification, the deprotonation of carbon dioxide is generally the most significant proton source (Table 2; cases 16, 18, 19, 20). This transfer of protons is greatly enhanced when the pH values of soil solutions approach or exceed the proton dissociation constant of CO₂ ($\text{pH} > \text{pK}_a = 6.3$). Dissolved organic acids can deprotonate at low pH ($\text{pK} \approx 4\text{--}5$) values as is apparent in the two bogs (cases 3 and 4) and two of the four podzols (cases 5 and 8) considered here. Note that transport of organic anions (A⁻ in Table 3) causes soil acidification only when cations are removed simultaneously with A⁻ in the drainage water. Transport of partly dissociated organic acids ($\text{HA} + \text{A}^- + \text{H}^+$) may cause relatively low pH values in the drainage water but has, in fact, no effect

on either ANC (s) or ANC (aq). Contrary to the contention of others^{3,4}, organic matter in the soil has no direct role in soil acidification, but merely acts as a (cation) exchanger. Effects of cation exchange on the proton budgets are accounted for under the category 'weathering'.

In terrestrial systems with acidic soils and low to intermediate rates of soil acidification, biologically mediated processes can contribute significantly to the internal production of protons. Most vegetation assimilates an excess of non-nitrogen cations over anions. In an aggrading ecosystem this process results in a net production of protons (cases 6–8, 10–14). In a few ecosystems (cases 9 and 21) biomass serves as a net proton sink due to net mineralization processes.

In a deforested watershed at the Hubbard Brook Experimental Forest (HBEF) in New Hampshire (case 21) a herbicide was applied for 3 yr to prevent vegetation regrowth in the deforested area while decomposition processes were allowed to proceed. Decomposition of the forest floor followed by nitrification resulted in excessive proton production. Levels of proton flux, largely resulting from these nitrogen transformations even exceeded some of our values for calcareous systems. Note that only a modest fraction (31%) of this proton loading was neutralized by weathering reactions while the balance of these protons was neutralized through net cationic decomposition of the forest floor and nitrate loss (denitrification?). In ambient conditions at the HBEF (case 7) proton neutralization is largely (88%) achieved by cationic weathering. While the deforestation experiment is an extreme example, it illustrates the nature of mineralization processes in proton transformations.

Biomass also indirectly regulates proton flux by reducing the flow of water through soil. Using data from Castricum, a cal-

careous site in the Netherlands, we computed proton budgets for sites under pine (*Pinus nigra*; case 20), oak (*Quercus robur*; case 19) and with no vegetation (case 18). Although the soils and geology at all three sites are similar, proton flux and rates of soil acidification were very different. Solute concentrations in drainage water were highest at the pine site, intermediate at the oak site and lowest at the unvegetated site. However, these trends in solute concentration were more than compensated for by variations in water flux. High rates of transpiration from the pine stand reduced the flow of water and solutes percolating through the soil and therefore reduced the rate of soil acidification. Drainage fluxes were intermediate at the oak stand and highest at the unvegetated site; the rates of soil acidification at these sites varied accordingly.

External proton sources

As the world average (case 1) and data from more or less pristine ecosystems (cases 2, 9, 15, 16) would attest, atmospheric inputs do not contribute significantly to the proton loading of these ecosystems. However, near centres of anthropogenic activity such as the northeastern portion of the USA (cases 3, 7) and in northwestern Europe (cases 4–6, 8, 10–14, 17–20), atmospheric proton inputs are substantial. For ecosystems with low to intermediate rates of soil acidification, external loading can be a major fraction of the total proton sources. In the USA (cases 3, 7) atmospheric proton inputs largely occur as free protons and to a lesser extent as SO_2 . In Central Europe dry deposition is a very significant mechanism of proton loading. At Hackfort (cases 12–14, 17) in the Netherlands transformations of atmospherically derived ammonium ($(\text{NH}_4)_2\text{SO}_4$), mainly from dry deposition, account for 53–66% of the total proton sources¹³. At Solling in West Germany (case 10, 11), the rates of soil acidification are much greater in a spruce site ($-5.9 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$) than in a beech site ($-2.9 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$). Spruce is more efficient than beech in scavenging sulphur from the atmosphere¹¹ (inputs due to dry deposition are $2.8 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$ in spruce compared with $0.9 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$ in beech) resulting in higher rates of soil acidification at the coniferous site.

Information on the input of external protons is insufficient to assess ecosystem sensitivity to acid inputs. Rather, a good measure of the capability of an ecosystem to withstand ambient external proton loading is the external/internal proton ratio (EIPR). For example, Castricum (cases 18–20) receives high atmospheric loadings of protons ($2.2\text{--}2.5 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$), but this external input is small relative to the internal proton sources in the calcareous soils ($\text{EIPR} = 0.13\text{--}0.26$). Such soils can easily accommodate this external proton load because soil acidification proceeds entirely through the dissolution of basic cations, mainly Ca^{2+} from CaCO_3 (Table 3).

Systems with low rates of soil acidification such as in Birkeness, Norway (case 5), Lüneburger Heide, West Germany (case 6), the HBEF (case 7), and Gårdsjön, Sweden (case 8) are extremely proton sensitive. These systems receive inputs of acidic and potentially acidic substances ($1.1\text{--}1.7 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$) that are comparable to internal proton sources ($\text{EIPR} = 0.5\text{--}2.4$). In these conditions soil acidification proceeds partly through the dissolution of aluminium minerals and significant levels of free protons and aluminium are present in the soil solutions and drainage waters.

In forests in central Europe (cases 10–14, 17, 19 and 20) external proton inputs are extremely high ($2.2\text{--}6.3 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$) as are EIPR values for ecosystems with low to intermediate rates of soil weathering (cases 10–14). Moreover, in these ecosystems the soil acidification process is characterized by aluminium dissolution as well as strong retention of sulphate. Appreciable quantities of hydrogen ion and aluminium are exported from the soil to the drainage water.

Note that because of very low rates of internal proton production, Thoreau's Bog (case 3) and Storgama (case 4) have high EIPR values. However, these systems may accommodate high loadings of atmospheric protons through anion assimilation by

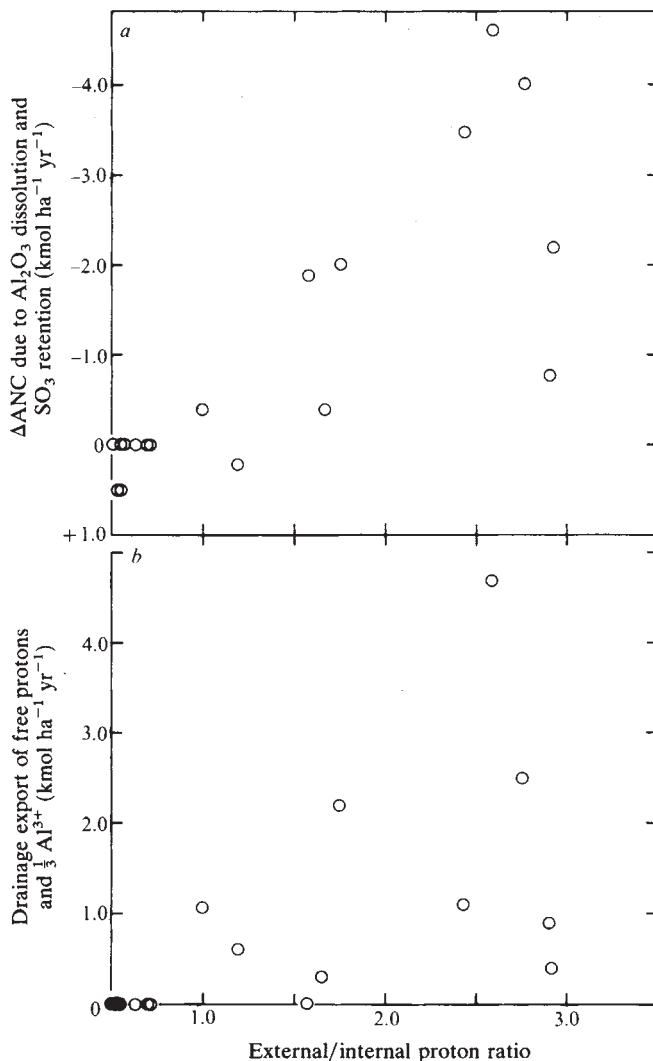


Fig. 1 The rate of soil acidification due to Al_2O_3 dissolution and SO_3 retention (a) and the drainage flux of BNC from the soil (b) as a function of the external/internal proton ratio of ecosystems. Data from bogs and deforested ecosystems are not included.

biomass or by microbially-mediated sulphate and nitrate reduction.

To assess the capacity of terrestrial ecosystems to assimilate ambient external acid inputs in an innocuous manner, we have plotted the rate of soil acidification due to Al_2O_3 dissolution and sulphate retention (Fig. 1a) and drainage flux of BNC (Fig. 1b) as a function of the EIPR. At low (<0.3) EIPR values ecosystems are able to assimilate proton inputs and soil acidification proceeds largely through the dissolution of basic cations. Most ecosystems with EIPR values above 0.5 show significant dissolution of soil aluminium and sulphate retention, resulting in export of free protons and aluminium in drainage waters.

Conclusions

In well drained soils in areas with excess precipitation over evapotranspiration, protons are generated by natural processes (mainly weak acid deprotonation and net assimilation of cations by the vegetation). Soil acidification results from removal of cations and retention of anions. In near-neutral soils rich in weatherable minerals, soil acidification is relatively rapid ($7.5\text{--}16 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$) and mainly associated with CO_2 deprotonation. In acidic soils, soil acidification is slower ($<2.5 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$) with cation assimilation and organic acid deprotonation as the principal internal H^+ sources. In the northeastern USA and central and northwestern Europe atmospheric deposition of anthropogenically-derived acidic substances (mainly H^+ , SO_2 and NH_4^+) may be $1\text{--}5 \text{ kmol hectare}^{-1} \text{ yr}^{-1}$.

This deposition exceeds internally generated protons in soils with low rates of soil acidification and in many soils with intermediate rates of soil acidification. As a result potentially toxic inorganic aluminium is released into the soil solution and H^+ - and aluminium base-neutralizing capacity is transported to drainage waters.

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1. Ulrich, B. & Matzner, E. *Abiotische Folgewirkungen der weiträumigen Ausbreitung von Luftverunreinigungen* (Luftreinhalte Forschungsbericht 10402615, Umweltbundesamt, 1983).
2. Overrein, L. N., Seip, H. M. & Tollan, A. (eds) *SNSF Proj. Res. Rep. FR 19/80* (1980).
3. Rosenqvist, I. Th. *Sur Jord-Surt Vann* (Ingeniør Forlaget A/S, Oslo, 1977).
4. Krug, E. C. & Frink, C. R. *Science* **221**, 520–525 (1983).
5. Van Breemen, N., Mulder, J. & Driscoll, C. T. *Pl. Soil* **75**, 283–308 (1983).
6. Driscoll, C. T. & Likens, G. E. *Tellus* **34**, 283–292 (1982).
7. Stumm, W. & Morgan, J. J. *Aquatic Chemistry* (Wiley, New York, 1970).
8. Van Breemen, N. & Wielemaker, W. G. *Proc. Soil Sci. Soc. Am.* **38**, 61–66 (1974).
9. Johnson, N. M., Driscoll, C. T., Eaton, J. S., Likens, G. E. & McDowell, W. H. *Geochim. cosmochim. Acta* **45**, 1421–1437 (1981).
10. Baker, J. & Schofield, C. *Water, Air Soil Pollut.* **18**, 289–309 (1982).
11. Ulrich, B., Mayer, R. & Khanna, P. K. *Soil Sci.* **130**, 193–199 (1980).
12. Ulrich, B., Mayer, R. & Khanna, P. K. *Deposition von Luftverunreinigungen und ihre Auswirkungen in Waldökosystemen im Solling* ((Sauerländer, Frankfurt a. M., 1979).
13. Van Breemen, N. *et al. Nature* **299**, 548–550 (1982).
14. KNMI/RIV-project Meetnet voor Bepaling van de Chemische Samenstelling van de Neerslag in Nederland Jaaroverzicht (KNMI, De Bilt, 1981).
15. Cole, D. W. & Rapp, M. in *Dynamic Properties of Forest Ecosystems* (ed. Reichle, D. E.) 341–409 (Cambridge University Press, 1981).
16. Ovington, J. D. & Madgwick, H. A. I. *For. Sci.* **5**, 344–355 (1959).

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17. Garrels, R. M. & MacKenzie, F. T. *Evolution of Sedimentary Rocks* (Norton, New York, 1971).
18. Carmouze, J. P. *La Régulation Hydrogéochimique du Lac Tchad* (ORSTOM, Paris, 1976).
19. Hemond, H. F. *Ecol. Monogr.* **50**, 507–526 (1980).
20. Hemond, H. F. *Ecology* **64**, 99–109 (1983).
21. Braekke, F. H. *Rep. Norwegian Forest Res. Inst.* **36.9** (1981).
22. Braekke, F. H. *Rep. Norwegian Forest Res. Inst.* **36.12** (1981).
23. Matzner, E. & Ulrich, B. *Z. Pfl.-Ernähr. Bodenkd.* **143**, 666–678 (1980).
24. Likens, G. E., Bormann, F. H., Pierce, R. S., Eaton, J. S. & Johnson, N. M. *Biogeochemistry of a Forested Ecosystem* (Springer, New York, 1977).
25. Hultberg, H. *Ecol. Bull.* **36** (in the press).
26. Bruynzeel, L. A. thesis, Free Univ. Amsterdam (1983).
27. Matzner, E. *et al. Elementflüsse in Waldökosystemen im Solling-Datendokumentation* (Göttinger Bodenkundliche Berichte 71, 1982).
28. Van Breemen, N., Van Grinsven, J. J. M. & Jordens, E. R. *Proc. Int. Conf. Acid Precipitation—Origin and Effects* (VDI Düsseldorf, in the press).
29. Sollins, P. *et al. Ecol. Monogr.* **50**, 261–285 (1980).
30. Reynolds, R. C. & Johnson, N. M. *Geochim. cosmochim. Acta* **36**, 537–554 (1972).
31. Minderman, G. & Leeftang, K. W. F. *Pl. Soil* **28**, 61–80 (1968).
32. Likens, G. E., Bormann, F. H., Johnson, N. M., Fischer, D. W. & Pierce, R. S. *Ecol. Monogr.* **40**, 23–47 (1970).
33. Bormann, F. H. & Likens, G. E. *Pattern and Process in a Forested Ecosystem* (Springer, New York, 1979).
34. Holland, H. D. *The Chemistry of the Atmosphere and Oceans* (Wiley, New York, 1978).

Expression of functional acetylcholine receptor from cloned cDNAs

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The cloned cDNAs encoding the four subunits of the Torpedo californica acetylcholine receptor, each carried by a simian virus 40 vector, direct the synthesis of the functional receptor in a combined expression system consisting of COS monkey cells and Xenopus oocytes. Our results suggest that all four subunits are required to elicit a normal nicotinic response to acetylcholine, whereas only the α -subunit is indispensable for α -bungarotoxin binding activity.

THE nicotinic acetylcholine receptor (AChR) is a postsynaptic membrane protein complex that alters the ionic permeability of the membrane as a consequence of binding acetylcholine. The AChR from the electric organ of the ray *Torpedo californica* is composed of five subunits present in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ and contains both the binding site for the neurotransmitter and the ionic channel (reviewed in refs 1–3). Recently, the primary structures of all four subunits of the *T. californica* AChR^{4–6} and the α -subunit of the calf and human muscle AChR⁷ have been elucidated in our laboratory by cloning and sequencing cDNAs or genomic DNA encoding these polypeptides; cDNA sequences for the γ -subunit of the *T. californica* AChR⁸ and the α -subunit of the *Torpedo marmorata* AChR^{9,10} have also been reported by other groups. On the basis of the amino acid sequences of the four subunits, the locations of functionally and immunologically important regions of the AChR molecule have been proposed. These include the acetylcholine binding site^{4–7}, the transmembrane segments^{4–8,10} presumably involved in the ionic channel, and the 'main immunogenic region'^{4,6,7} related causally to myasthenia gravis (reviewed in ref. 11).

One approach to verifying these proposed sites would be to introduce amino acid alterations into the respective regions of the AChR molecule and to analyse the resultant effects on the

receptor function and the antigenic determinants. As a first step towards this goal, we have now developed a combined system consisting of COS monkey cells and *Xenopus* oocytes in which the synthesis of the functional AChR is directed by the cloned cDNAs encoding the four subunits of the *T. californica* receptor, each carried by a simian virus 40 (SV40) vector. This expression system has allowed us to study the effect of deletion of each subunit on AChR function.

Construction of vectors

The expression vector used (pKCRH2), a derivative of the plasmid pKCR constructed by O'Hare *et al.*¹², contained the SV40 early gene promoter, donor and acceptor splice sites derived from the rabbit β -globin gene, and polyadenylation sites derived from the rabbit β -globin gene and the SV40 early gene. The entire protein-coding sequence of the cDNA for each subunit of the *T. californica* AChR^{4–6}, cloned into the Okayama-Berg vector¹³, was excised by cleavage at the *Hind*III or *Eco*RI site, which was introduced into both the 5'- and 3'-noncoding regions of each cDNA by using synthetic linkers (Fig. 1a). The excised cDNA fragment was inserted into the vector pKCRH2 at the *Hind*III site and/or the *Eco*RI site located between the acceptor splice site and the polyadenylation site derived from the rabbit β -globin gene. The structure of the

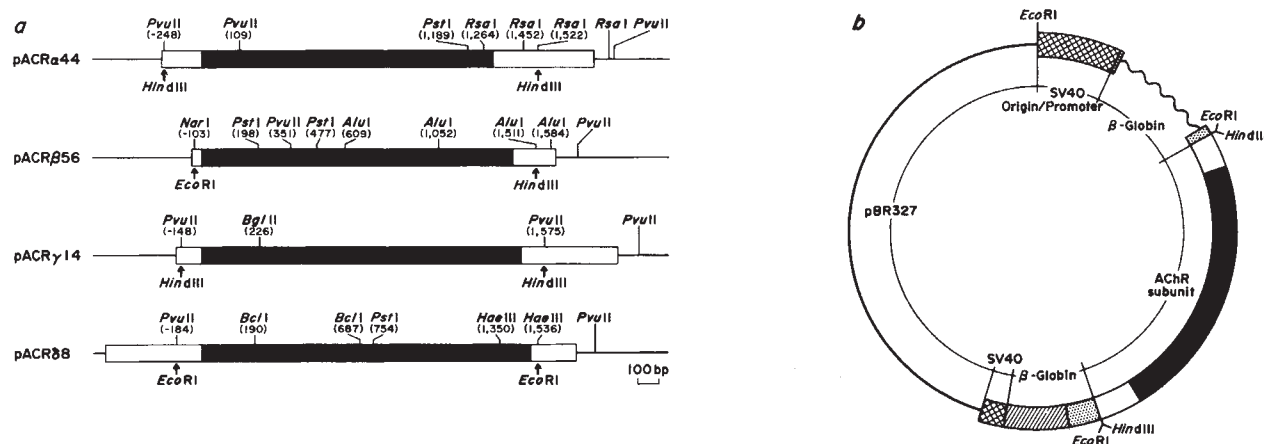


Fig. 1 Recombinant plasmids used for the expression of AChR. **a**, Restriction maps of the cloned AChR subunit cDNAs. The plasmids pACRα44, pACRβ56, pACRγ14 and pACRδ88, carrying cDNAs for the α-, β-, γ- and δ-subunits of the *T. californica* AChR, respectively, have been described previously⁴⁻⁶. The protein-coding regions are indicated by closed boxes, the 5'- and 3'-noncoding regions by open boxes, and the vector sequence including the tails by lines. Only the relevant restriction endonuclease sites are shown and identified by numbers indicating the 5'-terminal nucleotide generated by cleavage; for numbering of the nucleotide residues, see refs 4-6. The *Hind*III and *Eco*RI sites introduced by the use of synthetic linkers are shown by arrows beneath the noncoding regions. **b**, Schematic representation of pBKR327 recombinant plasmids containing the respective AChR subunit cDNAs (pKCRα2, pKCRβ2, pKCRγ2 and pKCRδ2). The origin of the segments is shown on the inner circle. The protein-coding region of the AChR subunit cDNA is indicated by a closed box, and its 5'- and 3'-noncoding regions by open boxes. The SV40 sequences are shown by cross-hatched boxes; the orientation of the early gene promoter is such that transcription occurs clockwise. The exonic sequences of the rabbit β-globin gene are indicated by stippled boxes, its intronic sequence by a wavy line, and its 3'-flanking sequence by a hatched box. The sequence of the plasmid pBKR327 is represented by a line. Only the relevant restriction endonuclease sites are shown.

Methods: The plasmid pKCRH2 was constructed as follows. The plasmid pKCR327 (given by C. Weissmann), in which the pBR322 sequence of pKCR (ref. 12) was replaced by the corresponding sequence of pBKR327 (ref. 31), was partially digested with *Eco*RI, treated successively by alkaline phosphatase and by T4 DNA polymerase with the four deoxyribonucleotide triphosphates (dNTPs), ligated to the *Hind*III linker dCAAGCTTG, cleaved with *Hind*III and ligated. One of the resulting plasmids, harbouring the *Hind*III site in the rabbit β-globin gene sequence, was designated pKCRH2. The pKCRH2 recombinants containing the respective AChR subunit cDNAs were constructed as follows. pACRα44 was partially digested with *Pvu*II, ligated to the *Hind*III linker and cleaved with *Hind*III and *Eco*RI, and the 4.4-kilobase pair (kb) fragment formed was isolated. The plasmid pSV2-gpt³² was cleaved with *Pvu*II, ligated to the *Eco*RI linker dGGAATTC and cleaved with *Eco*RI and *Hind*III, and the resulting 0.34-kb fragment containing the SV40 early gene promoter was isolated. The two DNA fragments thus obtained were ligated to yield the plasmid pSVACRα. pSVACRα was partially digested with *Pvu*II, ligated to the *Hind*III linker and cleaved with *Hind*III. The resulting 2.1-kb fragment containing the entire coding sequence for the AChR α-subunit was inserted into the *Hind*III site of pKCRH2 in the same orientation with respect to SV40 early gene transcription to generate the plasmid pKCRα1. The 0.70-kb *Pst*I-*Hind*III fragment derived from the 2.1-kb fragment described above was partially digested with *Rsa*I, ligated to the *Hind*III linker and cleaved with *Hind*III and *Pst*I. The resulting 0.34-kb fragment was isolated and ligated to the 4.1-kb *Hind*III-*Bam*HI fragment from pKCRH2 and the 2.1-kb *Bam*HI-*Pst*I fragment from pKCRα1, to yield pKCRα2. pACRβ56 was cleaved by *Eco*RI, treated successively with alkaline phosphatase and with T4 DNA polymerase with the four dNTPs and ligated to the *Eco*RI linker. After digestion with *Eco*RI, the 4.1-kb fragment formed was isolated and ligated. The resulting plasmid was partially cleaved with *Pvu*II, ligated to the *Eco*RI linker and cleaved with *Eco*RI. The resulting 1.8-kb fragment containing the entire coding sequence for the AChR β-subunit was inserted into the *Eco*RI site in the β-globin gene sequence of pKCR327 in the same orientation with respect to SV40 early gene transcription to produce the plasmid pKCRβ1. The 1.2-kb *Eco*RI-*Pst*I fragment derived from the 1.8-kb fragment described above was partially digested with *Alu*I, ligated to the *Hind*III linker and cleaved with *Hind*III and *Pst*I. The 1.0-kb fragment formed was isolated and ligated to the 4.1-kb *Bam*HI-*Hind*III fragment from pKCRH2 and the 1.2-kb *Bam*HI-*Pst*I fragment from pKCRβ1, to yield pKCRβ2. pACRγ14 was partially digested with *Pvu*II, ligated to the *Hind*III linker, cleaved with *Hind*III and partially digested with *Eco*RI. The resulting 4.5-kb fragment was ligated to the 0.34-kb *Eco*RI-*Hind*III fragment of pSVACRα to yield the plasmid pSVACRγ. pSVACRγ was partially digested with *Pvu*II, ligated to the *Hind*III linker and cleaved with *Hind*III. The resulting 2.2-kb fragment containing the entire coding sequence for the AChR γ-subunit was inserted into the *Hind*III site of pKCRH2 in the same orientation with respect to SV40 early gene transcription to generate the plasmid pKCRγ1. The 1.4-kb *Bgl*II-*Pvu*II fragment derived from the 2.2-kb fragment described above was ligated to the *Hind*III linker, digested with *Hind*III and *Bgl*II and ligated to the 2.9-kb *Hind*III-*Pst*I fragment from pKCRH2 and the 2.2-kb *Pst*I-*Bgl*II fragment from pKCRγ1, to yield pKCRγ2. pACRδ88 was digested with *Pvu*II, ligated to the *Eco*RI linker and cleaved with *Eco*RI. The resulting 2.0-kb fragment containing the entire coding sequence for the AChR δ-subunit was inserted into the *Eco*RI site in the β-globin sequence of pKCR327 in the same orientation with respect to SV40 early gene transcription, to produce the plasmid pKCRδ1. The 1.1-kb fragment formed by cleaving the 2.0-kb fragment described above with *Eco*RI, *Pst*I and *Bcl*I was isolated, partially digested with *Hae*III, ligated to the *Eco*RI linker and cleaved with *Eco*RI and *Pst*I. The resulting 0.79-kb fragment was isolated and ligated to the 4.1-kb *Eco*RI-*Bam*HI fragment from pKCR327 and the 1.6-kb *Bam*HI-*Pst*I fragment from pKCRδ1, to yield pKCRδ2. For further experimental details, see ref. 33.

recombinant plasmids thus constructed is schematically illustrated in Fig. 1b; the plasmids containing the α-, β-, γ- and δ-subunit cDNAs are designated pKCRα2, pKCRβ2, pKCRγ2 and pKCRδ2, respectively (see Fig. 1 legend for experimental details).

Expression of functional AChR

Because the recombinant plasmids described above contain the replication origin of SV40, they are expected to propagate to high copy numbers in COS monkey cells¹⁴, which produce SV40 T-antigen and are permissive for the replication of these recombinants. Therefore, each recombinant plasmid was introduced into COS cells by transfection¹⁵. S₁ nuclease mapping¹⁶ of poly(A) RNA isolated from the transfected cells indicated that the 5' end of the AChR subunit-specific mRNAs produced from the recombinant plasmids corresponded to the heterogeneous start sites of the transcripts formed under the control of the SV40 early gene promoter^{12,17-19} and that the intronic sequence of the rabbit β-globin gene was eliminated. Thus, the AChR

subunit-specific mRNAs produced in the transfected COS cells contained no AUG triplet upstream of the translation initiation site for the AChR subunit precursors⁴⁻⁶. The contents of the α-, β-, γ- and δ-subunit-specific mRNAs in poly(A) RNA from the transfected cells, as quantified by S₁ nuclease analysis, were respectively, 2.3-5.6 times, 2.6-6.8 times, 1.6-4.6 times and 0.9-1.4 times the level in *T. californica* electroplax poly(A) RNA.

To examine whether the AChR subunit-specific mRNAs produced from the recombinant plasmids could direct the synthesis of the functional AChR, we used the *Xenopus* oocyte translation system²⁰⁻²². The mRNAs specific for the α-, β-, γ- and δ-subunits of the AChR, derived from transfected COS cells, were combined in the same ratio as in *T. californica* electroplax RNA and were introduced into oocytes by microinjection. Extracts from the injected oocytes, prepared 3 days later, exhibited ¹²⁵I-α-bungarotoxin binding activity ranging between 0.3 and 3 fmol per oocyte (see Table 1 and Fig. 4 legend for experimental details).

The injected oocytes, after incubation for 3 days, were studied electrophysiologically by recording the intracellular potentials or the membrane currents under voltage clamp²². The response to acetylcholine applied ionophoretically was examined in the presence of atropine which blocks the native muscarinic AChR present in the membrane of *Xenopus* oocytes^{23,24}. Figure 2a shows that an inward current with a short latency was observed in response to acetylcholine. This response was virtually abolished within 3 min after the application of (+)tubocurarine (Fig. 2b), and the effect was reversible (Fig. 2c). The magnitude of the acetylcholine response depended on the membrane potential. The reversal potential of the acetylcholine response, measured in six injected oocytes under voltage clamp, was -5 to -1 mV. The reversal potential was not altered in a Cl^- -free solution. When the ionophoretic application of acetylcholine was repeated at short intervals (10–20 s), the response was progressively diminished because of desensitization of the AChR. These results indicate that *Xenopus* oocytes injected with the four AChR subunit-specific mRNAs from transfected COS cells synthesize the functional AChR of the nicotinic type which increases the cationic conductances in response to acetylcholine. The electrophysiological properties of the AChR formed by translation of the mRNAs derived from the recombinant plasmids are in good agreement with those of AChR synthesized in oocytes injected with *Torpedo* electroplax poly(A) RNA²².

The translation products encoded by the AChR subunit-specific mRNAs from transfected COS cells were identified by labelling them in oocytes with ^{35}S -methionine and analysing them by SDS-polyacrylamide gel electrophoresis after α -toxin affinity chromatography and immunoprecipitation. Figure 3a shows that oocytes injected with these mRNAs (lanes 6–8), like oocytes injected with *T. californica* electroplax poly(A) RNA (lanes 3–5), contained four polypeptides that were specifically precipitated with antiserum to the *T. californica* AChR and that were indistinguishable with respect to mobility from the subunits of the purified native AChR (lane 2) except for the γ -subunit. The γ -subunit synthesized in both groups of oocytes migrated somewhat faster than that of the purified native AChR; this may be due to a difference in glycosylation or other processing events *in vivo* or to proteolytic modification during the isolation procedure¹.

Effect of deletion of each subunit

In view of the conspicuous amino acid sequence homology found among the four subunits of the AChR and of their pseudosymmetric orientation across the membrane, proposed on the basis of their similarity in hydrophilicity profile and predicted secondary structure^{4–6}, the question arises as to whether or not all four subunits are required for receptor function. The expression system described above allowed us to examine this issue.



Fig. 2 Acetylcholine responses recorded for a *Xenopus* oocyte injected with the four AChR subunit-specific mRNAs produced from the recombinant plasmids in COS cells. The recordings were made at room temperature in a frog Ringer's solution containing $1 \mu\text{M}$ atropine: upper traces, currents used for ionophoresis of acetylcholine (backing current, 12 nA); lower traces, acetylcholine currents recorded under voltage clamp (downward deflection indicates inward current). The membrane potential was held at -60 mV , and acetylcholine was applied to the vegetal pole. Responses to acetylcholine before (a) and 2.5 min after perfusion with $2.5 \mu\text{M}$ (+)tubocurarine (b), and 15 min after washing out the antagonist (c).

Methods: COS monkey cells¹⁴ were transfected with pKCR α 2, pKCR β 2, pKCR γ 2 or pKCR δ 2 ($20 \mu\text{g}$ of each recombinant DNA per 90-mm dish without carrier DNA) as described previously³³. After 42–48 h, the cells were collected and total RNA was extracted as described elsewhere³⁴, except that the homogenization buffer contained 0.5% Nonidet P-40. Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography³⁵. About 20 ng of poly(A) RNA, containing the α -, β -, γ - and δ -subunit-specific mRNAs, were injected into each oocyte of *Xenopus laevis*. The injected oocytes were incubated at 19°C for 3 days in modified Barth's medium²⁰ containing gentamicin (0.1 mg ml^{-1}) and nystatin (50 U ml^{-1}). Oocytes having a healthy appearance were selected for subsequent studies.

Oocytes were injected with various combinations of three of the AChR subunit-specific mRNAs, and the resulting translation products were first analysed to ensure that the subunit polypeptides encoded by the three mRNAs injected were actually formed. For this purpose, the translation products, labelled in oocytes with ^{35}S -methionine, were subjected to immunoprecipitation with a mixture of four monoclonal antibodies²⁵, each specific for the individual subunits, and then to electrophoretic analysis. The results showed that every combination of three of the mRNAs introduced into oocytes directed the synthesis of the corresponding subunits, although the amounts of the polypeptides found were variable, depending on the particular mRNA combinations (Fig. 3b). In comparison with a control experiment in which all four mRNAs were injected (lane 2), smaller amounts of the respective subunits were generally present in oocytes when only three of the mRNAs were injected. Densitometric scanning of the fluorogram indicated that the levels of the α - and δ -subunits were especially low, being $\sim 4\%$

Table 1 Effect of deletion of each subunit on AChR function

AChR subunit-specific mRNAs injected	^{125}I - α -bungarotoxin binding† (fmol per oocyte)	Response to acetylcholine*	
		No. of responsive oocytes/no. of oocytes tested	Mean acetylcholine sensitivity‡ (mV μC^{-1})
$\alpha, \beta, \gamma, \delta$	2.06	126/170	37 (0.4–460)
β, γ, δ	<0.01	0/82	—
α, γ, δ	0.06	0/82	—
α, β, δ	0.09	2/82	1 (0.9–1)
α, β, γ	0.33	3/105	2 (0.2–4)
None	<0.01	0/80	—

Oocytes were injected with various combinations of the AChR subunit-specific mRNAs as indicated and were incubated for 3 days as described in Fig. 2 legend. The ^{125}I - α -bungarotoxin binding activity in the cell extract from each group of oocytes (24–110 oocytes per assay) was determined by the antibody precipitation assay³⁰ using a mixture of the four subunit-specific monoclonal antibodies (see Fig. 3 legend); incubation was at room temperature for 3 h in the presence of 5 nM ^{125}I - α -bungarotoxin (230 Ci mmol^{-1}). Acetylcholine potentials were recorded at a holding potential of $\sim -70 \text{ mV}$; see Fig. 2 legend for further details.

* Our detectable limit of acetylcholine sensitivity was $\sim 0.2 \text{ mV } \mu\text{C}^{-1}$.

† Means from assays of 106–343 oocytes.

‡ The means were calculated only for the responsive oocytes; the ranges of acetylcholine sensitivity are given in parentheses.

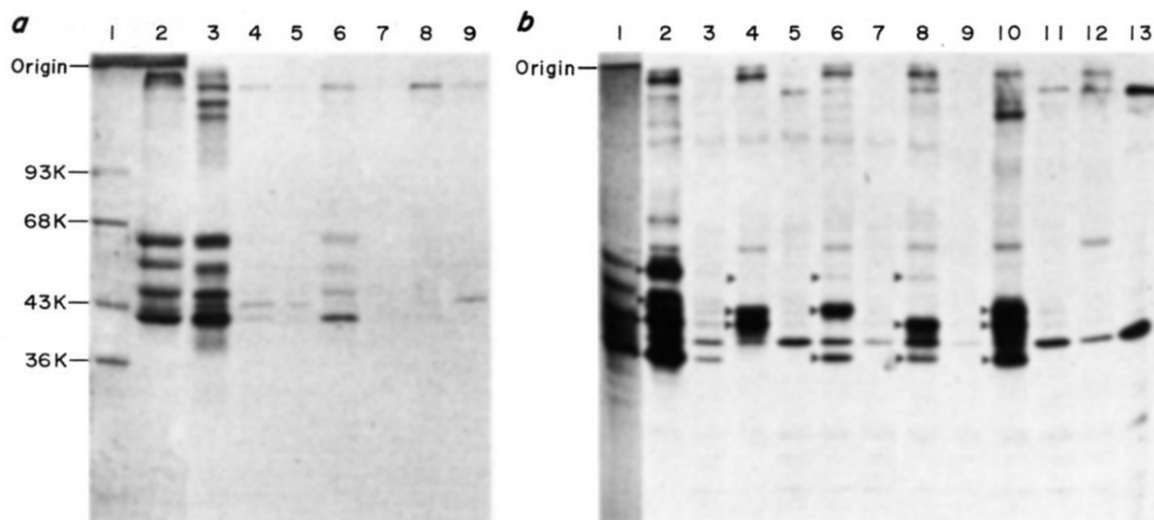


Fig. 3 Electrophoretic analysis of the translation products encoded by AChR subunit-specific mRNAs produced from recombinant plasmids in COS cells. Oocytes were injected with various combinations of the AChR subunit-specific mRNAs from transfected COS cells or with *T. californica* electroplax poly(A) RNA^{35,36}, then were incubated for 3 (a) or 2 (b) days in the presence of L-[³⁵S]methionine (1,440 Ci mmol⁻¹; final concentration 1 mCi ml⁻¹). The homogenate²¹ of the oocytes was centrifuged at 100,000g for 60 min, and the supernatant (cell extract) was subjected to the following procedures. In a, each cell extract was shaken with *Laticauda semifasciata* III-Sepharose. After washing, the AChR was eluted with 1 M carbamylcholine, immunoprecipitated with rabbit antiserum to intact *T. californica* AChR and protein A-Sepharose, and electrophoresed on a SDS/10% polyacrylamide gel³⁷. The fluorograms³⁸ of the electrophoretic analyses (lanes 3–9), together with the standard polypeptides stained with Coomassie brilliant blue (lanes 1 and 2), are shown. Lane 1, size markers: rabbit lactate dehydrogenase [M_r 36,000 (36K)], chicken ovalbumin (M_r 43K), bovine serum albumin (M_r 68K) and rabbit muscle phosphorylase b (M_r 93K). Lane 2, α -, β -, γ - and δ -subunits (from bottom to top) of native *T. californica* AChR purified by α -toxin affinity chromatography³⁹. Lane 3, AChR preparation derived from 33 oocytes injected with *T. californica* electroplax poly(A) RNA. Lane 4, a control for lane 3 (immunoprecipitate obtained in the presence of a large excess of native *T. californica* AChR). Lane 5, another control for lane 3 (immunoprecipitate obtained with non-immunized rabbit serum). Lane 6, AChR preparation derived from 100 oocytes injected with the α -, β -, γ - and δ -subunit-specific mRNAs. Lane 7, a control for lane 6 (like lane 4). Lane 8, another control for lane 6 (like lane 5). Lane 9, immunoprecipitate derived from 100 oocytes injected with poly(A) RNA from non-transfected COS cells. In b, 51 ng of purified native *T. californica* AChR as carrier was added to each homogenate derived from 80 injected oocytes, and each cell extract was subjected to prior treatment with non-immunized rabbit serum and protein A-Sepharose to diminish co-precipitation of nonspecific proteins. Immunoprecipitation was done using a mixture of the four subunit-specific monoclonal antibodies²⁵ (2.6 pmol each of monoclonal antibodies (against intact AChR) mAb5, mAb111, mAb154 and mAb137 specific for the α -, β -, γ - and δ -subunits of the *T. californica* AChR, respectively) and anti-rat immunoglobulin-Sepharose. The resulting immunoprecipitates were electrophoresed on a SDS/10% polyacrylamide gel: lane 1, subunits of native *T. californica* AChR stained; lanes 2, 4, 6, 8, 10 and 12, fluorograms of the electrophoretic analyses of the immunoprecipitates derived from oocytes injected with the α -, β -, γ - and δ -subunit-specific mRNAs (lane 2), the β -, γ - and δ -subunit-specific mRNAs (lane 4), the α -, γ - and δ -subunit-specific mRNAs (lane 6), the α -, β - and δ -subunit-specific mRNAs (lane 8), the α -, β - and γ -subunit-specific mRNAs (lane 10) and poly(A) RNA from non-transfected COS cells (lane 12). Lanes 3, 5, 7, 9, 11 and 13 are controls for lanes 2, 4, 6, 8, 10 and 12, respectively (immunoprecipitates obtained in the presence of a large excess of native *T. californica* AChR). The AChR subunits derived from the oocytes are indicated by arrowheads.

(lanes 6 and 8) and ~1% (lane 4) or ~3% (lanes 6 and 8) of the control value, respectively, except for the α -subunit (~20%) found in the absence of the δ -subunit (lane 10). On the other hand, the levels of the β -subunit (lanes 4, 8 and 10) and the γ -subunit (lanes 4, 6 and 10) were relatively high, being ~30 to ~80% of the control value. These observations suggest that the subunit polypeptides, when one of them is lacking, are more readily degraded in oocytes or during the isolation procedure, although the possibility of different rates of their synthesis cannot be excluded. The absence of one subunit may hinder the assembly of the remaining subunits or lead to the formation of an aberrant complex, thus reducing the stability of the constituent polypeptides. It is interesting in this context that, in the absence of the δ -subunit, fairly large amounts of the remaining three subunits, including the otherwise unstable α -subunit, were found. Thus, the coexistence of the β - and γ -subunits seems to stabilize the α -subunit, presumably by forming a complex of these three subunits with a relatively correct conformation. On the other hand, the δ -subunit seems to be stabilized only when all of the three other subunits are present.

In the experiments shown in Table 1, oocytes injected with various combinations of three of the AChR subunit-specific mRNAs were tested for ¹²⁵I- α -bungarotoxin binding activity in the cell extract as well as for the response of the membrane

potential to acetylcholine. The cell extracts containing all but the β -, γ - or δ -subunit exhibited 3, 4 and 16%, respectively, of the α -bungarotoxin binding activity of the complete AChR synthesized in oocytes, whereas the cell extract containing all but the α -subunit showed no detectable activity. The extent of α -bungarotoxin binding seems to correlate with the amount of the α -subunit present (see above and Fig. 3b). This is consistent with the finding that the isolated α -subunit alone is capable of binding α -bungarotoxin^{26,27}.

The cell extract containing all four subunits and that containing all but the δ -subunit, which exhibited a relatively high level of α -bungarotoxin binding activity, were tested for the protective effect of an agonist on α -toxin binding²⁸. Figure 4 shows that, in the presence of carbamylcholine at increasing concentrations, the ¹²⁵I- α -bungarotoxin binding activity in both of the cell extracts decreased gradually. The carbamylcholine concentration required for 50% protection of the complete AChR was essentially the same as that observed for the native *T. californica* AChR, suggesting that the AChR encoded by the mRNAs produced from the recombinant plasmids possesses an agonist binding site having normal affinity. An approximately fivefold higher concentration of carbamylcholine was required for 50% protection of the AChR lacking the δ -subunit; the apparent binding affinity of α -bungarotoxin was lower for the

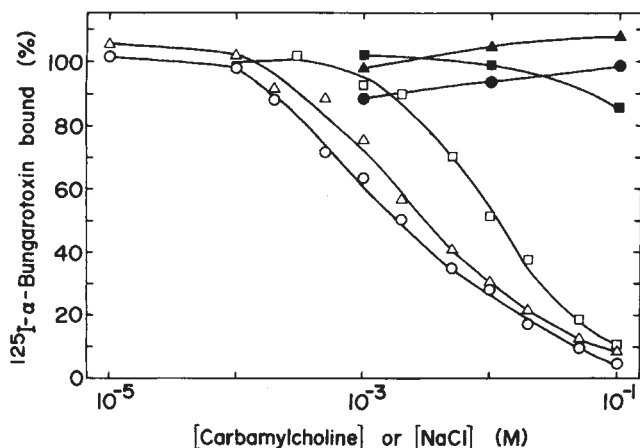


Fig. 4 Effect of carbamylcholine on ^{125}I - α -bungarotoxin binding. Cell extracts prepared from oocytes injected as indicated, or purified native *T. californica* AChR dissolved in the homogenization buffer²¹, were preincubated at room temperature for 10 min with carbamylcholine at the specified concentrations and were then mixed with ^{125}I - α -bungarotoxin (230 Ci mmol⁻¹; final concentration 7 nM); the concentration of the toxin-binding site was 63–78 pM. After incubation at room temperature for 3 h, the amount of the toxin bound was determined by the antibody precipitation method³⁰ using rabbit antiserum to native *T. californica* AChR. ○, Extract from oocytes injected with the α -, β -, γ - and δ -subunit-specific mRNAs from transfected COS cells (●, control experiment in which carbamylcholine was replaced by NaCl at the indicated concentrations). □, Extract from oocytes injected with the α -, β - and γ -subunit-specific mRNAs from transfected COS cells (■, control experiment as above). ▲, Purified native AChR (▲, control experiment as above). The apparent K_D value for ^{125}I - α -bungarotoxin, estimated by varying the toxin concentration at a fixed concentration (20–43 pM) of the toxin-binding site⁴⁰, was 0.1 nM for the native AChR, 0.2 nM for the AChR from oocytes injected with the four subunit-specific mRNAs and 0.7 nM for the AChR from oocytes injected with the α -, β - and γ -subunit-specific mRNAs.

AChR lacking the δ -subunit than for the complete AChR (see Fig. 4 legend). These results imply that the agonist binding site is formed even in the absence of the δ -subunit, but its binding affinity is lower than in the intact AChR molecule.

Of the oocytes injected with all four AChR subunit-specific mRNAs, 74% responded to acetylcholine, when tested electrophysiologically (Table 1). In contrast, the oocytes injected with three of the mRNAs exhibited no acetylcholine potential, except that 2–3% of the oocytes injected with all but the γ - or

δ -subunit-specific mRNA showed a weak (+) tubocurarine-sensitive response. The mean acetylcholine sensitivity²⁹ of the responsive oocytes, expressed as the acetylcholine potential amplitude (mV) divided by the quantity of electricity (μC) used for ionophoresis of acetylcholine, was $37 \text{ mV } \mu\text{C}^{-1}$ for the oocytes with the complete AChR, but was $1\text{--}2 \text{ mV } \mu\text{C}^{-1}$ for those with AChR lacking the γ - or δ -subunit.

In view of the low levels of the α - and δ -subunits found in the oocytes injected with three of the AChR subunit-specific mRNAs (see above and Fig. 3b), we examined the effect of reducing the amount of a mixture of the four mRNAs injected into oocytes on their responsiveness to acetylcholine. When oocytes were injected with a mixture of the four mRNAs diluted 10-fold with poly(A) RNA from non-transfected COS cells, 59% of the oocytes (22 out of 37) still responded to acetylcholine with a mean sensitivity of $3 \text{ mV } \mu\text{C}^{-1}$ (range $0.3\text{--}14 \text{ mV } \mu\text{C}^{-1}$), but the ^{125}I - α -bungarotoxin binding activity as well as the amounts of the α - and δ -subunits found in the oocytes treated in the same manner ranged between barely detectable levels and ~4% of the values obtained for the oocytes injected with a normal mixture of the four mRNAs. Therefore, the amounts of the individual subunits present in the oocytes injected with all but the β -, γ - or δ -subunit-specific mRNA (~3 to ~40%, ~3 to ~30% and ~20 to ~80% of the control value, respectively) would be sufficient for many of these oocytes to respond to acetylcholine. The amount of the δ -subunit present in the oocytes injected with all but the α -subunit-specific mRNA (~1% of the control value) might be limiting, but it is hardly conceivable that the AChR lacking the α -subunit can be functional because this subunit is principally responsible for binding acetylcholine¹.

Thus, our results suggest that all four subunits are required for normal AChR function. The possibility remains that the γ - and δ -subunits can replace each other to some extent for the receptor function because 2–3% of the oocytes injected with all but the γ - or δ -subunit-specific mRNA responded to acetylcholine, albeit slightly. However, the aberrant presence of the nicotinic AChR in non-injected oocytes cannot completely be excluded. The expression system described here will be useful for studying the structure–function relationship as well as the antigenic determinants of the AChR by introducing mutations into this molecule.

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- Karlin, A. in *Cell Surface Reviews* Vol. 6 (eds Cotman, C. W., Poste, G. & Nicolson, G. L.) 191–260 (North-Holland, Amsterdam, 1980).
- Changeux, J.-P. *Harvey Lect.* **75**, 85–254 (1981).
- Conti-Tronconi, B. M. & Raftery, M. A. *A. Rev. Biochem.* **51**, 491–530 (1982).
- Noda, M. *et al. Nature* **299**, 793–797 (1982).
- Noda, M. *et al. Nature* **301**, 251–255 (1983).
- Noda, M. *et al. Nature* **302**, 528–532 (1983).
- Noda, M. *et al. Nature* **305**, 818–823 (1983).
- Claudio, T., Ballivet, M., Patrick, J. & Heinemann, S. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1111–1115 (1983).
- Sumikawa, K. *et al. Nucleic Acids Res.* **10**, 5809–5822 (1982).
- Devillers-Thiery, A., Giraudat, J., Bentabollet, M. & Changeux, J.-P. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2067–2071 (1983).
- Lindstrom, J. & Dau, P. A. *Rev. Pharmac. Tox.* **20**, 337–362 (1980).
- O'Hare, K., Benoist, C. & Breathnach, R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1527–1531 (1981).
- Okayama, H. & Berg, P. *Molec. cell. Biol.* **2**, 161–170 (1982).
- Gluzman, Y. *Cell* **23**, 175–182 (1981).
- Graham, F. L. & van der Eb, A. J. *Virology* **52**, 456–467 (1973).
- Berk, A. J. & Sharp, P. A. *Cell* **12**, 721–732 (1977).
- Haegeman, G. & Fiers, W. J. *Virology* **35**, 955–961 (1980).
- Benoist, C. & Chambon, P. *Nature* **290**, 304–310 (1981).
- Gruss, P. & Khoury, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 133–137 (1981).

- Gurdon, J. B. *The Control of Gene Expression in Animal Development* (Clarendon, Oxford, 1974).
- Sumikawa, K., Houghton, M., Emtage, J. S., Richards, B. M. & Barnard, E. A. *Nature* **292**, 862–864 (1981).
- Barnard, E. A., Miledi, R. & Sumikawa, K. *Proc. R. Soc.* **215**, 241–246 (1982).
- Kusano, K., Miledi, R. & Stinnakre, J. *Nature* **270**, 739–741 (1977).
- Kusano, K., Miledi, R. & Stinnakre, J. *J. Physiol., Lond.* **328**, 143–170 (1982).
- Gullick, W. J. & Lindstrom, J. M. *Biochemistry* **22**, 3312–3320 (1983).
- Haggerty, J. G. & Froehner, S. C. *J. biol. Chem.* **256**, 8294–8297 (1981).
- Tzartos, S. J. & Changeux, J.-P. *EMBO J.* **2**, 381–387 (1983).
- Weber, M. & Changeux, J.-P. *Molec. Pharmacol.* **10**, 15–34 (1974).
- Miledi, R. *J. Physiol., Lond.* **151**, 1–23 (1960).
- Weinberg, C. B. & Hall, Z. W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 504–508 (1979).
- Soberon, X., Covarrubias, L. & Bolivar, F. *Gene* **9**, 287–305 (1980).
- Mulligan, R. C. & Berg, P. *Science* **209**, 1422–1427 (1980).
- Mishina, M. *et al. EMBO J.* **1**, 1533–1538 (1982).
- Nakamura, M., Nakanishi, S., Sueoka, S., Imura, H. & Numa, S. *Eur. J. Biochem.* **86**, 61–66 (1978).
- Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408–1412 (1972).
- Nakanishi, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 4319–4323 (1976).
- King, J. & Laemmli, U. K. *J. molec. Biol.* **62**, 465–477 (1971).
- Chamberlain, J. P. *Analyt. Biochem.* **98**, 132–135 (1979).
- Kaneda, N., Tanaka, F., Kohno, M., Hayashi, K. & Yagi, K. *Archs Biochem. Biophys.* **218**, 376–383 (1982).
- Weber, M. & Changeux, J.-P. *Molec. Pharmacol.* **10**, 1–14 (1974).

Two configurations of a channel-forming membrane protein

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The protein oligomer forming the gap junction channel has been analysed in two Ca^{2+} -sensitive states by electron microscopy of membranes in frozen aqueous solutions. Switching between states occurs by a small cooperative rearrangement involving tilting of the subunits, which may be responsible for the effect of Ca^{2+} on channel permeability in vivo.

FUNDAMENTAL biological processes such as the transmission of nerve impulses, visual excitation and cell-to-cell communication depend on the fact that membranes can regulate the passage of ions and/or small molecules across their surfaces. Proteins create channels in the membranes for this purpose and regulation is achieved by conformational changes of the protein in response to chemical or electrical stimuli. An example of such a protein is the one which forms the gap junction (or cell-to-cell) channel. We report here on its structure and action when stimulated by Ca^{2+} , an ion which induces this channel to close^{1,2}.

The protein is a cylindrical oligomer composed of six identical, rod-shaped subunits^{3,4}. It spans the lipid bilayer, creating a channel along its central, hexad, axis. The channel traverses the plasma membranes of two apposed cells in consequence of a hexamer in one membrane being joined symmetrically to an oppositely facing hexamer in the other. These 12-subunit assemblies accumulate at specialized regions of contact (gap junctions), where they regulate the passage of small molecules and ions between cell interiors (reviewed in refs 5, 6).

Based on the appearance of gap junction surfaces in negative stain, we speculated that the channel might open and close, given the appropriate stimulus, by a coordinated tilting and sliding motion of the protein subunits along their lines of contact⁴. Subsequent X-ray diffraction experiments showed that a reversible transition of this nature could be induced by the action of Ca^{2+} *in vitro*⁷. Now we describe the two Ca^{2+} -sensitive configurations seen more directly in electron micrographs of junctions in frozen aqueous solutions. The three-dimensional maps obtained by this technique^{8,9} reveal features of the subunits extending over their entire length, both within the lipid bilayer and in the aqueous spaces on either side. Differences between these maps demonstrate that Ca^{2+} does induce a small coordinated rearrangement of the subunits around the channel and affect its dimensions in a manner consistent with the original proposal.

Imaging and diffraction

We made one preparation of rat liver junctions for these experiments and produced two alternative structures by dialysing separate portions against Ca^{2+} -free (5 mM EGTA, 0.5 mM MgCl_2 , 5 mM HEPES pH 8.0) and Ca^{2+} -containing (0.05 mM CaCl_2 , 5 mM HEPES pH 8.0) solutions. Methods were as described previously⁷. Such junctions are double membrane plaques, 0.5–1.5 μm in diameter, composed of ordered domains of oppositely facing hexamers and intervening lipid molecules. The hexamers pack within the domains on a hexagonal lattice of dimension 80–85 Å and protrude into the space between the two membranes, making a gap between them of ~30 Å.

Electron microscopy was used to derive a set of three-dimensional Fourier terms corresponding to each structure. The junctions were frozen in their respective solutions and examined with a cold stage at -120°C . Images were recorded of specimens tilted at angles of up to 53° to the incident 100-kV electron beam (Fig. 1). Densitometry and computer processing were

carried out as described previously^{4,10} and the Fourier terms to 20 Å resolution from each image were combined according to the symmetry of the space group P6. The average phase errors in combining the data were 16.7° and 15° for the $-\text{Ca}^{2+}$ and $+\text{Ca}^{2+}$ structures, respectively, based on comparisons between each new phase and all previously accumulated values related by symmetry. For both structures, 18 images provided sufficient values of amplitude and phase to map out the continuous variations along each of the five crystallographically independent reciprocal lattice lines. Curves were fitted to the data along the lattice lines by a least-squares method¹¹, with the amplitude curves weighted according to the reliability of the corresponding phases (Fig. 2). These curves, sampled at regular intervals ($0.002 \text{ \AA}^{-1}$), provided the Fourier terms for calculating three-dimensional maps.

There were cones of missing information due to the restricted ranges of tilt. By far the most significant omissions, according to X rays⁷, were the terms from the central (0, 0) lattice line. These terms determine only the absolute levels of the contours in sections parallel to the membrane and therefore have no influence on the positions of peaks identifying the paths taken by the protein subunits, described below. They do, however, affect the dimensions of features bound by shallow gradients, for example the channel. Such dimensions were obtained more accurately previously⁴ with complete sets of Fourier terms.

X-ray diffraction was used (as in ref. 7) to provide an independent evaluation of the structures. The partially oriented gap junction pellets gave rise to intensities along the lattice lines which were smeared into concentric arcs. Traces across these arcs, obtained by circular integration, were very similar to analogous profiles calculated from the electron microscopic data (Fig. 2, panel 6). Moreover, in comparing the two structures,

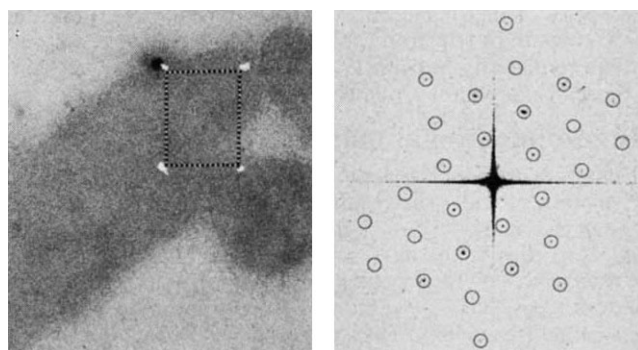


Fig. 1 Image ($\times 32,000$) and optical diffraction pattern (from the boxed area) of a gap junction frozen in Ca^{2+} -free solution and tilted 31.5° to the incident electron beam. The regular hexagonal lattice is evident from the array of diffraction peaks encircled, but is barely visible in the image because of the small differences in electron scattering properties between ice, lipid and protein.

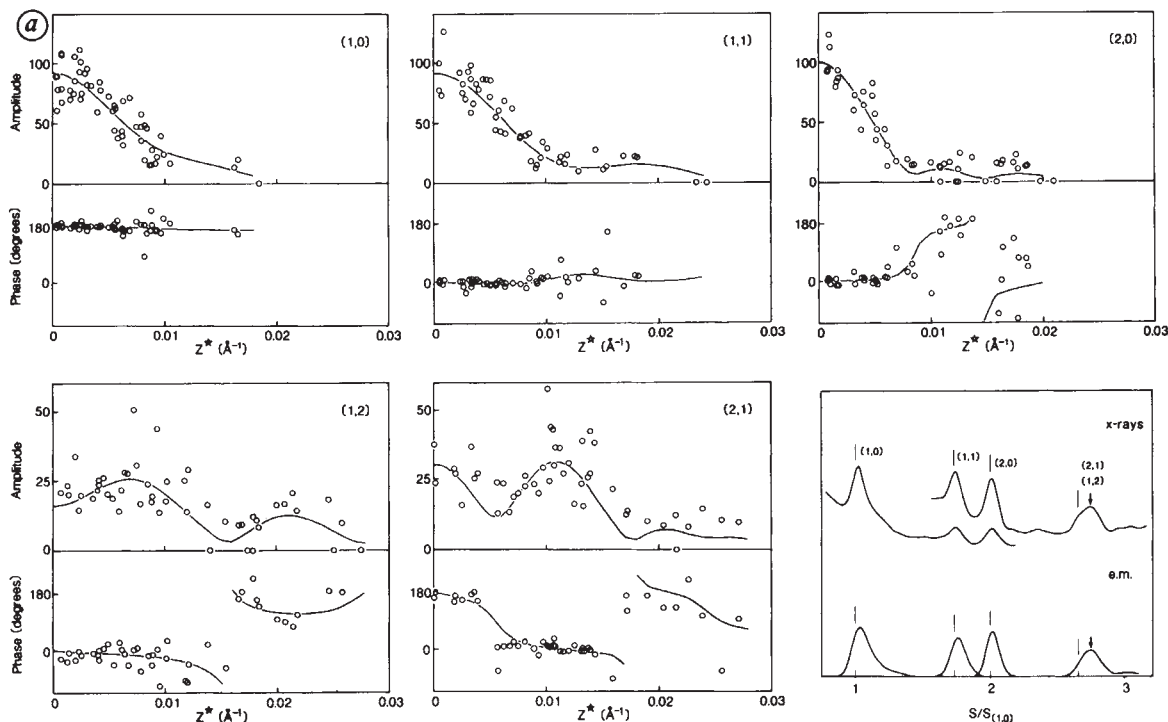


Fig. 2 Diffraction data from gap junctions in: *a*, Ca^{2+} -free solution; *b* (right), Ca^{2+} -containing solution. The values of amplitude and phase along the reciprocal lattice lines (first five panels) were derived from images of junctions tilted to the incident electron beam by angles of up to 53° in *a* and 50° in *b*. Z^* is the distance from the midpoint along the lattice line; the Friedel relationship gives the values for negative Z^* . The sixth panel shows X-ray traces.

Methods: Specimens were prepared by the double carbon film technique^{8,20}. The carbon support grid containing the junctions was immersed in $10\times$ diluted Ca^{2+} -free or Ca^{2+} -containing solution, raised through a second carbon film floated on the surface, pressed firmly onto Millipore filter paper, held away from the filter paper for several seconds and then plunged into liquid nitrogen. Holes in the support film were important for efficient withdrawal of excess solution onto the filter paper. After transfer to the microscope, the grids were maintained at $\sim -120^\circ\text{C}$ using a modified Philips EM300 high-resolution stage²¹. Images were focused using an off-axis viewing screen²², and recorded at a magnification of $\times 28,000$ on Kodak 4463 film with low doses ($2\text{--}5$ electrons $\text{\AA}^2$) to minimize radiation damage. Films were developed in full strength Kodak D19 developer for 8 min. Tilting was accomplished by bending the microscope grids under liquid nitrogen before inserting them into the stage. Preliminary selection of images was by optical diffraction evaluation of the strength of the Fourier terms and of the focus levels indicated by the positions of the zeros between the Thon rings¹⁹. Images only gave suitable diffraction patterns if they were of specimens surrounded by visible films of ice. Those images selected for subsequent processing and incorporation into the three-dimensional data sets all had underfocus values within the range: $4,000\text{--}12,000$ $\text{\AA}$; thus, variations in strengths of the Fourier terms due to different contrast transfer functions were small. In the processing, the densitometer step and sampling sizes were 20 μm , equivalent to 7 $\text{\AA}$ at the specimen, and the array size for Fourier transformation was 512×512 . An analysis of images very close to focus indicated that the poorer contrast transfer at low spatial frequencies, produced by underfocusing, weakened the amplitude of the $(1,0)$ peak by up to 30% relative to values for the other peaks; the same correction was applied to all images for this effect. Tilt angles and axes were estimated from the geometry of the peaks in the computed Fourier transforms and from focus changes across the film. In combining the Fourier terms from different images, the comparison range used along Z^* was 0.0022 $\text{\AA}^{-1}$. Images were not incorporated into the three-dimensional data sets if the phase residuals, based on comparisons with the previously accumulated phases, were greater than 25° . With specimens tilted by $>45^\circ$, some of the terms were omitted from the refinement. Unlike previously⁴, junctions from this preparation were strongly chiral (see also ref. 23), a feature most obvious from the opposite phases of the $(1,2)$ and $(2,1)$ peaks at low values of Z^* . Seven of the 18 images for *a* and 8 of the 18 images for *b* were of junctions with their positive crystallographic direction pointing towards the electron source. The X-ray traces (panel 6) were obtained from partially oriented pellets, as described previously⁷. The dimensionless parameter, $S/S_{(1,0)}$, provides a linear reciprocal scale such that the hexagonal lattice positions, marked by vertical lines, are at 1 , $\sqrt{3}$, 2 and $\sqrt{7}$. Peak values depart from these positions depending on the strength of the Fourier terms along the lattice lines away from the point, $Z^*=0$. The electron microscopy-derived curves compared were obtained by plotting the amplitudes against reciprocal spacing, rather than Z^* , squaring to obtain intensities and convolving these intensities with a gaussian distribution to simulate the broadening of the X-ray peaks due to finite beam size and disorder. Changes in the position of the overlapped $(2,1)$ and $(1,2)$ peak (arrows) and in the intensity of the $(1,0)$ peak represent the most significant differences between the two structures. These changes are consistently observed in the X-ray patterns⁷.

the same differences (displacement of the overlapped $(2,1)$ and $(1,2)$ peak; change in intensity of the $(1,0)$ peak) were indicated by X rays as by electrons. Therefore, we presume that the protein configurations being visualized at -120°C are the same as those at more natural temperatures.

Three-dimensional maps

The maps, synthesized from the two sets of Fourier terms, depict the protein subunits surrounding the channels and extending through the lipid bilayer into the aqueous spaces on either side (Fig. 3). The subunits have several features of interest. They are rods which in cross-section must taper significantly towards the hexad axis. They are tilted tangentially and radially around the channel (see below). They appear more distinct and slightly thicker in the bilayer than in the extracellular region, as if their secondary structures in the two environments are quite different.

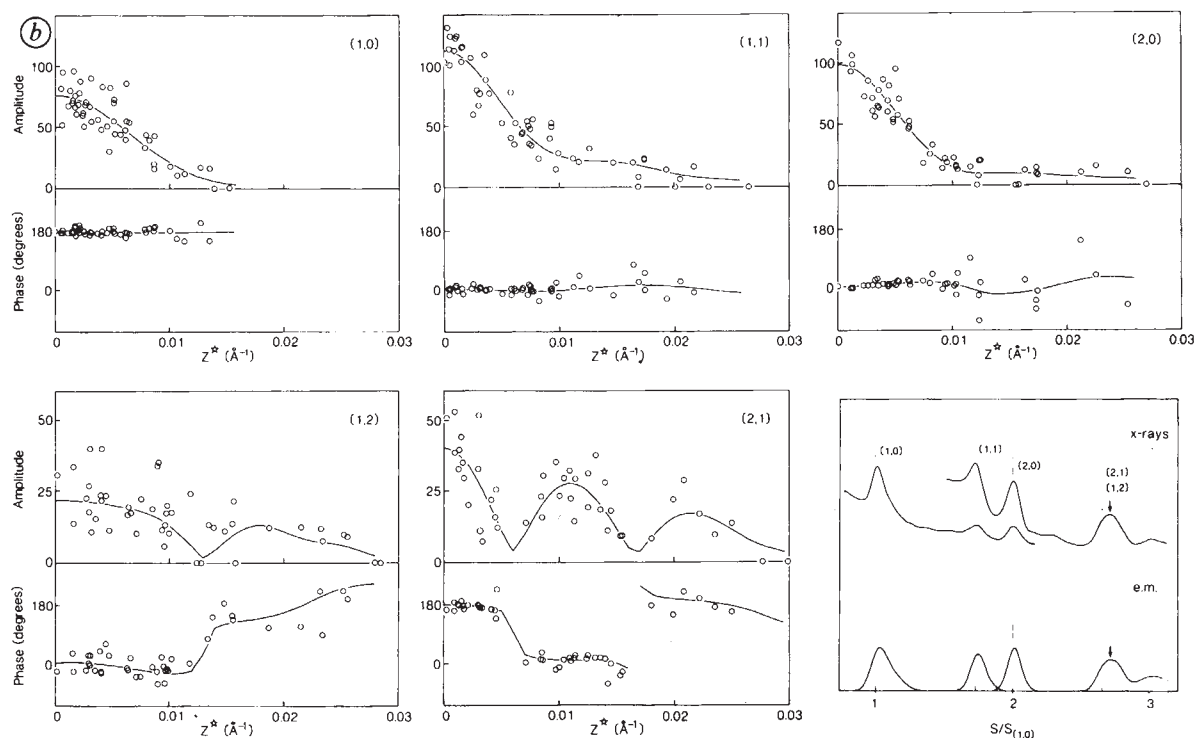
Ice and lipid are not separately resolved because of their similar mean electron scattering densities. Thus, the maps do not reveal accurately the extents to which the subunits protrude from the bilayer. However, the surface details seen with negative stain⁴ correspond to features in Fig. 3 and suggest that the

subunits protrude from the bilayer only slightly on the cytoplasmic side ($5\text{--}10$ $\text{\AA}$) compared with the extracellular side ($15\text{--}20$ $\text{\AA}$).

The hexamers come closest to each other inside the bilayer and the way they fit into the crystalline lattice depends on the bilayer in question. In Fig. 3 the ridges on the sides of the hexamers interlock, forming the same neighbour-to-neighbour relationship in both maps, while in the other bilayer they abut. The latter hexamers are less well preserved, and probably distorted, as the density fluctuations associated with their subunits are weaker (by 45% and 15% for the $-\text{Ca}^{2+}$ and $+\text{Ca}^{2+}$ structures, respectively). These effects may reflect constraints on the potentially more open packing due to the restricted volume of lipid present or due to the curved membranes of isolated junctions¹² being forced to lie flat. More symmetrical packing⁴ presumably occurs when more lipid is available to fill intervening spaces.

Differences

The hexamers look different in the two maps because of the different subunit configurations. They also have a slightly smaller



diameter in the $+Ca^{2+}$ structure, where the lattice dimension is almost 2 Å less⁷. We analysed the differences by comparing the tangential and the radial displacements of the peaks around the channel at various levels through the two structures. In projection down the hexad axis (Fig. 4) these peaks follow curved paths which are more foreshortened for the $+Ca^{2+}$ than for the $-Ca^{2+}$ subunits. The $+Ca^{2+}$ subunits, then, are aligned more nearly parallel to the hexad axis. From the direction and magnitude of foreshortening we find that the two paths of the subunits are related, approximately, by a tilt of $\sim 7.5^\circ$ tangential to the hexad axis and about the extracellular end (compare Fig. 4b and c).

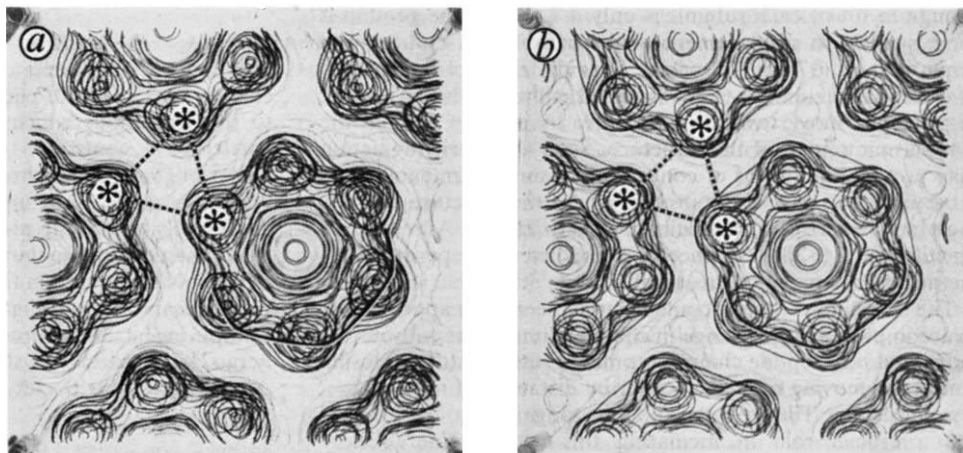
The radial path changes of the subunits in going to the $+Ca^{2+}$ configuration are small: about 2 Å inwards towards the cytoplasmic end, but outwards towards the extracellular end (Fig. 5). As shown, the constricting action at the cytoplasmic end would be expected from a tilt strictly tangential to the hexad axis. However, the opposite action at the other end is an additional feature, increasing slightly the separation between neighbouring subunits in the part of the structure outside the cell. This could be needed for the subunits to fit into the 'tighter' configuration without being deformed.

The significance of the small radial changes was checked by calculating difference Fourier maps between the two structures. In these maps the displacement of matter into and away from the channel is seen as two peaks of opposite sign, centred on the hexad axis and near the two ends of the subunits. The peaks were present in both membranes and did not depend on which way up one structure was with respect to the other. Further, the major difference terms responsible for the peaks were significant at the 5% level, based on the standard deviations of the observed amplitudes along the contributing reciprocal lattice lines.

Switching between the two structures

The three-dimensional maps revealed several basic features of this channel-forming protein. It has a length of ~ 70 Å perpendicular to the plane of the membrane and an average diameter of ~ 64 Å within this plane. It occupies a space of $\sim 225,000$ Å³ (from the zero-level contour), consistent with the value estimated from the subunit molecular weight, 28,000 daltons (refs 13, 14), assuming 1.3 Å³ per dalton for the partial specific volume. It is composed of two main portions, one within and the other outside the membrane, which differ distinctly in

Fig. 3 Maps of the $-Ca^{2+}$ (a) and $+Ca^{2+}$ (b) structures in one of the membranes, as they would appear viewed from the inside of the cell. The distance between channels in a is 82.4 Å and in b it is 80.6 Å, from X rays⁷. The six surrounding protein subunits create strong fluctuations in density within the lipid bilayer, but weaker fluctuations in the aqueous extracellular space (furthestmost portion of hexamer, outer surface partly outlined). All hexamers have the same neighbour-to-neighbour relationship where they come closest to each other, near the middle of the bilayer (indicated by equivalent triangles connecting equivalent points in the two maps). The positive and zero-level contours (darker) correspond to regions of higher electron scattering density. Detail extends over a distance of 68 Å perpendicular to the sections; fluctuations in density outside the maps on the cytoplasmic side are less than one contour level.



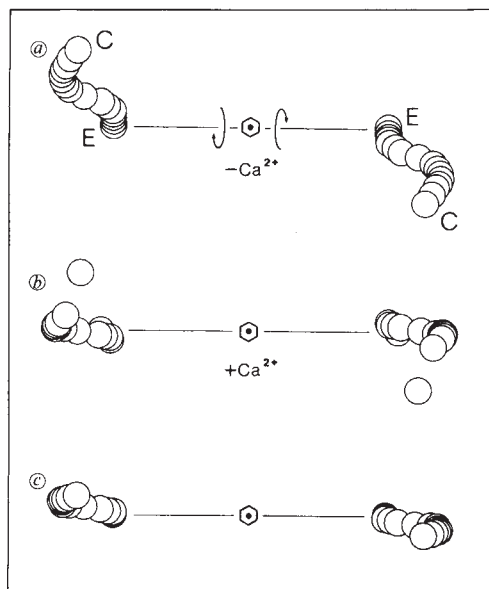


Fig. 4 Projection down the hexad axis of paths taken by subunits on opposite sides of the channel: *a*, $-Ca^{2+}$ and *b*, $+Ca^{2+}$ structures; *c*, path derived from *a* assuming a tilt of 7.5° about the radial axis is indicated. The circles mark the peak positions in sections parallel to the membrane through the three-dimensional maps. E and C are the extracellular and cytoplasmic ends of the subunits, respectively, corresponding to the top and bottom sections in Fig. 3. Correspondence between *c* and *b* indicates that the paths in *a* and *b* are related, approximately, by a small tilt tangential to the hexad axis.

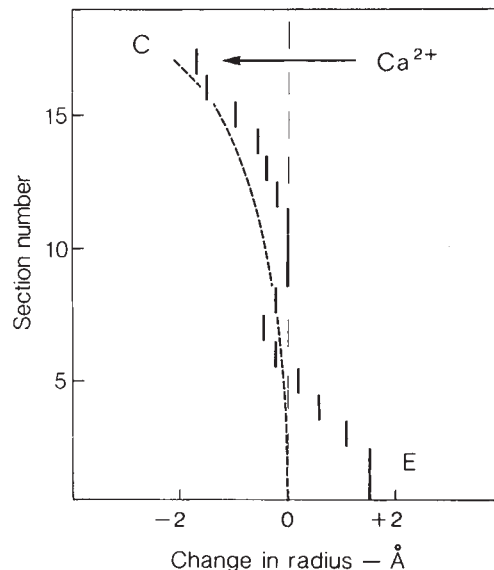


Fig. 5 Radial displacements of the subunits relative to the hexad axis (vertical) on addition of Ca^{2+} , determined from the positions of peaks around the channel in sections through the three-dimensional maps. The broken line shows the radial displacement corresponding to the tilt *a* to *c* in Fig. 4. E and C are as in Fig. 4. Similar radial displacements are suggested by the negative stain maps (ref. 4, Fig. 4), which show a decrease and an increase in stain accumulation in the cytoplasmic and extracellular parts of the channel, respectively.

appearance. In detail, the structure appears rather different from that determined from mouse liver gap junctions^{3,12}.

Calcium acts to produce small variations in these features by changing, or switching, the configurations of the subunits. From the differences in configuration investigated above, the switch must entail motion of essentially rigid rods having surfaces which do not rotate about the rod axes, but continue to point in the same directions. At low resolution, therefore, it consists simply of a rearrangement of the subunits by tilting and sliding along their lines of contact. This observed mechanism by which the protein changes its structure confirms the one proposed⁴, based on the appearance of gap junction surfaces in negative stain.

What is the effect of calcium on the dimensions of the channel? The rearrangement is depicted by wooden models (Fig. 6). We found that subunits on either side of the channel become aligned more nearly parallel to it on exposure to Ca^{2+} . They tilt in opposite senses about a radial axis at the extracellular end (bottom of model) and consequently are displaced tangentially towards each other at the cytoplasmic end (top of model). The change in tilt of each subunit is only $\sim 7.5^\circ$, but the protein is long, so that its displacement at the cytoplasmic end is quite large, that is, $\sim 70 \sin 7.5 = 9 \text{ \AA}$. Thus the channel-lining faces of each pair of subunits (not visualized at this resolution) could, in principle, move towards each other by up to $\sim 18 \text{ \AA}$ in the cytoplasmic region of the structure. Such a constricting action may provide the means of controlling channel permeability, as it occurs in the part of the protein where the structure changes most and where the channel appears to close⁴. A restrictive opening of $16\text{--}20 \text{ \AA}$ diameter is predicted by permeability measurements made on mammalian cells^{15,16}.

The switching between configurations seen here appears able to accomplish a large change in channel dimensions without the individual polypeptide chains becoming grossly distorted, losing contact or moving over each other by distances of more than a few angstroms. Tilting of the subunits tangential to the channel has a crucial role in mediating this action. Energetically unfavourable interactions are avoided by the subunits moving predominantly parallel to the plane of the bilayer and keeping the same polar and hydrophobic surfaces exposed to the solvent

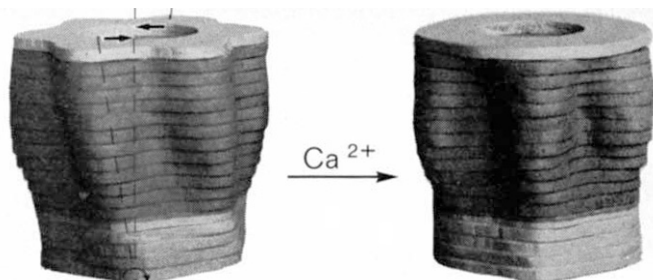


Fig. 6 Models of the two protein configurations, constructed from the maps in Fig. 3. The lighter shaded portions correspond to parts of the protein contacting fluid inside (top) and outside (bottom) the cell; the darker shaded portion would be in contact with lipid. The configurations are related to each other by tilt axes passing radially through the bases of the subunits (as depicted on the left-hand model). Subunits on either side of the channel tilt in opposite directions about these axes, and the resulting tangential displacements (pair of arrows) are greatest at the upper, cytoplasmic face.

and lipid. It is a cooperative mechanism, as the subunits must change their mode of packing together, and may be analogous to the transitions which occur in spherical and helical virus particles^{17,18}, controlled by divalent cation concentration or pH. Other oligomeric membrane proteins may respond to chemical or electrical stimuli by undergoing similar rearrangements predominantly within the plane of the bilayer.

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1. Rose, B. & Loewenstein, W. R. *Nature* **254**, 250–252 (1975).
2. Spray, D. C., Stern, J. H., Harris, A. L. & Bennett, M. V. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 441–445 (1981).

3. Makowski, L., Caspar, D. L. D., Phillips, W. C. & Goodenough, D. A. *J. Cell Biol.* **74**, 629–645 (1977).
4. Unwin, P. N. T. & Zampighi, G. *Nature* **283**, 545–549 (1980).
5. Hertzberg, E. G., Lawrence, T. S. & Gilula, N. B. *A. Rev. Physiol.* **43**, 479–491 (1981).
6. Loewenstein, W. R. *Physiol. Rev.* **61**, 829–913 (1981).
7. Unwin, P. N. T. & Ennis, P. D. *J. Cell Biol.* **97**, 1459–1466 (1983).
8. Taylor, K. A. & Glaeser, R. M. *Science* **186**, 1036–1037 (1974).
9. Dubochet, J., Lepault, J., Freeman, R., Berriman, J. A. & Homo, J.-C. *J. Microsc.* **128**, 219–237 (1982).
10. Henderson, R. & Unwin, P. N. T. *Nature* **257**, 28–32 (1975).
11. Agard, D. A. *J. molec. Biol.* **167**, 849–852 (1983).
12. Caspar, D. L. D., Goodenough, D. A., Makowski, L. & Phillips, W. C. *J. Cell Biol.* **74**, 605–628 (1977).

13. Hertzberg, E. L. & Gilula, N. B. *J. biol. Chem.* **254**, 2138–2147 (1979).
14. Nicholson, B. J., Hunkapiller, M. W., Grim, L. B., Hood, L. E. & Revel, J.-P. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7594–7598 (1981).
15. Flagg-Newton, J., Simpson, I. & Loewenstein, W. R. *Science* **205**, 404–407 (1979).
16. Schwarzmann, G. *et al. Science* **213**, 551–553 (1981).
17. Durham, A. C. H. & Klug, A. *Nature new Biol.* **229**, 6–10 (1971).
18. Robinson, I. K. & Harrison, S. C. *Nature* **297**, 563–568 (1982).
19. Thon, F. Z. *Naturforsch.* **219**, 476–478 (1966).
20. Jaffe, J. & Glaeser, R. M. *Proc. 40th an. EMSA Meet.*, 72–73 (San Francisco, 1982).
21. Taylor, K. A., Milligan, R. A., Raeburn, C. & Unwin, P. N. T. *Ultramicroscopy* (submitted).
22. Unwin, P. N. T. & Henderson, R. *J. molec. Biol.* **95**, 425–440 (1975).
23. Baker, T. S., Caspar, D. L. D., Hollingshead, C. J. & Goodenough, D. A. *J. Cell Biol.* **96**, 204–216 (1983).

LETTERS TO NATURE

Implications of 10^{16} eV γ rays from Cyg X-3

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Samorski and Stamm¹ have reported and Lloyd-Evans *et al.*² have recently confirmed the detection of high-energy quanta, presumably γ rays, with energies $E > 2 \times 10^{15}$ eV from Cygnus X-3. These ultra-high energy (UHE) γ rays were detected with extensive air shower arrays and included four events with $E > 10^{16}$ eV. Temporal analyses^{1,2} of the events indicate that the flux is modulated with a 4.8-h period and is sharply pulsed. Here (1) we discuss the implications of these γ -ray detections and suggest that autocorrelating the air shower data may be the best way to determine the intrinsic width of the γ -ray pulses; (2) we argue that the radiating particles are accelerated by a pulsar and that if they are accelerated according to any pulsar mechanism we know of, then they must be ions; (3) we note that if the ions are accelerated to 10^{16} eV by a large amplitude Deutsch wave, then the gravitational wave luminosity L_g should exceed that of the Crab pulsar by a factor of $\sim 5 \times 10^5$, and the spin-down time should be ~ 80 yr (requiring a truly remarkable object); and (4) we show that the ions can be accelerated in the near zone but only if, contrary to the standard view, pair production does not greatly reduce the vacuum potential drop in the near zone. We note that near-zone acceleration could be confirmed by detection of curvature radiation.

Several groups have detected γ rays from the Cyg X-3 system at energies $< 10^{15}$ eV using atmospheric Cerenkov techniques. The system is known to be a luminous ($L_{\text{peak}} > 10^{37}$ erg s⁻¹) source of TeV γ rays^{3–5}. The flux at TeV energies is periodic and has the same 4.8-h periodicity seen in medium energy γ rays⁶ ($\sim 10^2$ MeV), X rays⁷, and at IR energies⁸ and is generally identified as the orbital period. The TeV light curve is composed of two narrow pulses separated by ~ 1.9 h (ref. 3), and roughly centred about the X-ray minimum. A possible detection of γ rays having energies in the range 10^{14} – 10^{15} eV is reported by Bhat *et al.*⁹, who studied the arrival times of atmospheric Cerenkov pulses produced by air showers with energies $E > 10^{14}$ eV. By autocorrelating their data they found a significant excess of events with time separations $\Delta t < 40$ s that had a sidereal association. The width of the directional enhancement peak in right ascension is comparable to the angular response function of the detection system. Their data is consistent with a point source at a right ascension of 20 ± 1 h. The detection system was stationed at Gulmarg, India (latitude 34° N) and pointed at the zenith. It is tempting to identify this nonrandom

air shower component with γ rays from Cyg X-3 ($\alpha = 20$ h 31 min, $\delta = 40^\circ 47'$). However, the required flux would have to be roughly a factor of three larger than the upper limit of Fegan and Danaher¹⁰ and several orders of magnitude higher than the value obtained by interpolating between measurements at 10^{15} eV and below 10^{13} eV. Nevertheless, we feel that autocorrelating the data may be the best way to determine the intrinsic width of the γ -ray pulses since small errors in the ephemeris will broaden pulses in phase histograms that are generated using data obtained over long periods of time. Taken at face value, the observations of Bhat *et al.*⁹ indicate an excess only for $\Delta t < 40$ s (the autocorrelation was done only up to $\Delta t = 450$ s) and require the pulses to be extremely narrow ($\Delta\phi = 2 \times 10^{-3}$). We feel that an autocorrelation analysis of data obtained by a detection system with better angular resolution could be useful for determining whether or not the pulses are actually this narrow.

A natural interpretation of the narrow pulses is that Cyg X-3 is a young pulsar in a binary system¹¹ that is emitting UHE particles which interact with the corona of the companion star¹². Most of the UHE emission should come from a translucent 'window' (optical depth $\tau \sim 1$) in the companion star's corona, which may be extremely variable and complex due to its interaction with the pulsar wind. Higher τ would obscure the photons, and lower τ would be inefficient as a scintillation screen. It seems reasonable that the translucency condition is fulfilled, during any given orbital period, for 40 s which would account for the autocorrelation time scale. If the pulse width is actually this narrow, the intrinsic luminosity of the system must be $L > 1.3 \times 10^{39}$ erg s⁻¹; this would rank the pulsar as the youngest known.

The production of γ rays with $E > 10^{16}$ eV requires, of course, the acceleration of particles to even higher energies. We have argued elsewhere¹² that shock-accelerated particles could not generate narrow γ -ray pulses at energies $E > 10^{14}$ eV. The basic point is that shock acceleration requires scattering, whereas the narrow pulses at the observed phases imply straight-line trajectories. Additional arguments rule out shock acceleration of electrons beyond $\sim 10^{14}$ eV (ref. 12).

We now consider whether acceleration by a large amplitude Deutsch wave, as proposed by Gunn and Ostriker¹³, could account for particles whose energy exceeds 10^{16} eV. The maximum energy that a particle with mass Am_p and charge Ze can attain by this mechanism is

$$E_{\text{max}} \approx 6 \times 10^{14} A^{1/3} Z^{2/3} \times \left(\frac{B_{\text{surf}}}{10^{12} \text{ G}} \right)^{2/3} \left(\frac{P}{10 \text{ ms}} \right)^{-4/3} \left[\ln \left(\frac{R_B}{R_L} \right) \right]^{2/3} \text{ eV} \quad (1)$$

where B_{surf} is the surface magnetic field, P is the pulsar's period, and R_L and R_B are the radii of the light cylinder and binary orbit, respectively. The luminosity in magnetic dipole radiation would be (magnetic moment assumed $\perp$ to $\hat{\Omega}$):

$$L_{\text{MD}} \approx 6.5 \times 10^{40} \left(\frac{P}{10 \text{ ms}} \right)^{-4} \left(\frac{B_{\text{surf}}}{10^{12} \text{ G}} \right)^2 \text{ erg s}^{-1} \quad (2)$$

so the maximum particle energy is given by

$$E_{\max} \approx 6.0 \times 10^{14} A^{1/3} Z^{2/3} \times \left(\frac{L_{\text{MD}}}{6.5 \times 10^{40} \text{ erg s}^{-1}} \right)^{1/3} \left(\ln \left(\frac{R_B}{R_N} \right) \right)^{2/3} \text{ eV} \quad (3)$$

If the 10^{16} eV particles are electrons or positrons, then equation (3) implies that $L_{\text{MD}} > 2 \times 10^{46} \text{ erg s}^{-1}$, and the gravitational wave luminosity $L_g (\propto \Omega^6)$, is even larger for any reasonable estimate of the quadrupole moment. However, the maximum rotational energy is $\sim 10^{52} \text{ erg}$, and Cyg X-3 has been observed for over a decade with no obvious change in any of its behaviour, so the total luminosity must be $< 10^{43} \text{ erg s}^{-1}$. This implies that particles accelerated to 10^{16} eV would have to be ions.

If ions are accelerated to $E > 10^{16}$ eV by a large amplitude Deutsch wave, the pulsar should be a strong source of gravitational radiation. Using equations (1) and (3) we find that if the accelerated ions are protons, the required period is $\sim 3.7 (B_{\text{surf}}/10^{12} \text{ G})^{1/2} \text{ ms}$ and the corresponding magnetic dipole luminosity is $L_{\text{MD}} > 3 \times 10^{14} \text{ erg s}^{-1}$. Defining the quadrupole moment D as being equal to $q(6 \times 10^{41}) (B_{\text{surf}}/10^{12} \text{ G})^2$, for easy comparison with Gunn and Ostriker¹³, the spin-down rate due to gravitational radiation is $5q^2 (B_{\text{surf}}/10^{12} \text{ G})^{-1} \times 10^{-8} \text{ s}^{-1}$. Requiring the spin-down rate to be comfortably less than $(10 \text{ yr})^{-1}$ limits q to less than $\sim 0.25 (B_{\text{surf}}/10^{12} \text{ G})^{-1}$. The corresponding gravitational wave luminosity would be $L_g \approx 5 \times 10^{43} q^2 (B_{\text{surf}}/10^{12} \text{ G})^{-1} \text{ erg s}^{-1}$, larger than that from the Crab pulsar by 5×10^5 . This may be detectable, even without knowing the precise period, with a relatively low Q bar antenna. A young luminous pulsar in a close binary system like Cyg X-3 would probably drive a wind off the companion star that would enshroud the entire system; to this we attribute the lack of any detected pulses thus far. Indeed, X-ray observations¹⁴ and radio observations¹⁵ indicate that the system is embedded in optically thick material. In any case, the extreme youth of the pulsar required by the mechanism's production of 10^{16} eV ions could be considered an uncomfortably strong assumption.

An alternative view, which may be slightly less radical, is that the $> 10^{16}$ eV particles are accelerated in the near zone along polar field lines where charge-separated zones can generate large potential drops¹⁶⁻¹⁸. A very liberal estimate of the maximum potential drop $\Phi_{\max}$ is found by assuming that the entire polar zone is evacuated and the full vacuum potential is available¹⁶. In this case,

$$\Phi_{\max} \approx 7 \times 10^{16} \left(\frac{B_{\text{surf}}}{10^{12} \text{ G}} \right) \left(\frac{P}{10 \text{ ms}} \right)^2 \left(\frac{R}{10 \text{ km}} \right)^3 \text{ V} \quad (4)$$

A pulsar in the Cyg X-3 system comparable to (perhaps slightly younger than) the Crab could account for 10^{16} eV particles if the full vacuum potential were available. However, near-zone acceleration requires that the particles be ions, as curvature radiation from electrons travelling along field lines (with radius of curvature R_c) limits the electron energy to

$$E_{\max} \approx 1.5 \times 10^{13} \left(\frac{P}{10 \text{ ms}} \right)^{-1/4} \times \left(\frac{R_c}{10 \text{ km}} \right)^{1/2} \left(\frac{B_{\text{surf}}}{10^{12} \text{ G}} \right)^{1/4} \text{ eV} \quad (5)$$

Moreover, the UHE observations imply that pair production does not significantly short out electric fields in the near zone. Neither of these conclusions is expected from standard pulsar theory.

The possible detection^{19,20} of $E > 10^{15}$ eV γ rays from the Crab could be explained as being curvature radiation from 10^{16} eV ions that are accelerated in the near zone. A better detection of this source—and similarly, the detection of a steady component of 10^{16} eV γ rays from Cyg X-3—would independently support the model of ion acceleration in the near zone.

This would seem at present to be the least radical account of the 10^{16} eV γ rays that have been reported.

The more recent reports (ref. 21) of 10^{17} eV air showers from the direction of Cygnus cannot be accounted for by either of the pulsar mechanisms discussed here, when parameters that are consistent with a spin-down time $> 10 \text{ yr}$ are used. If this directional excess is real and is found to have a 4.8-h period, more exotic models for particle acceleration in the Cyg X-3 system are required.

In conclusion, the detections of $> 10^{15}$ eV γ rays from Cyg X-3 and their possible detection from the Crab have important implications for the theory of pulsars.

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- Samorski, M. & Stamm, W. *Astrophys. J. Lett.* **268**, L17-L22 (1983).
- Lloyd-Evans, J. *et al. Nature* **305**, 784-787 (1983).
- Nesphore, Yu I. *et al. Astrophys. Space Sci.* **61**, 349-355 (1979).
- Danaher, S., Fegan, D., Porter, N. & Weekes, T. *Nature* **289**, 568-569 (1981).
- Lamb, R., Godfrey, C., Wheaton, W. & Tumer, T. *Nature* **296**, 543-544 (1982).
- Lamb, R., Fichtel, C., Hartman, R., Kniffen, D. & Thomson, D. *Astrophys. J. Lett.* **212**, L63-L66 (1977).
- Parsignault, D., Grindlay, J., Gursky, H. & Tucker, W. *Astrophys. J.* **218**, 232-242 (1977).
- Becklin, E. *et al. Nature* **245**, 302-304 (1973).
- Bhat, C., Sparu, M. & Kaul, C. *Nature* **288**, 146-149 (Corrigendum: **291**, 168) (1980).
- Fegan, D. J. & Danaher, S. *Proc. 17th int. Cosmic Ray Conf.* **1**, 31-33 (1981).
- Milgrom, M. & Pines, D. *Astrophys. J.* **220**, 272-278 (1982).
- Vestrand, W. T. & Eichler, D. *Astrophys. J.* **261**, 251-258 (1982).
- Gunn, J. E. & Ostriker, J. P. *Phys. Rev. Lett.* **22**, 728-731 (1969).
- Hertz, P., Joss, P. & Rappaport, S. *Astrophys. J.* **224**, 614-624 (1978).
- Vestrand, W. T. *Astrophys. J.* **271**, 304-314 (1983).
- Arons, J. in *IAU Conf. No. 94*, 175-204 (Reidel, Dordrecht, 1981).
- Ruderman, M. A. & Sutherland, P. G. *Astrophys. J.* **196**, 51-72 (1975).
- Sturrock, P. A. *Astrophys. J.* **164**, 529-556 (1971).
- Boone, J. *et al.* preprint University of Utah (1983).
- Dzikowski, T., Gawin, J., Grochalska, B., Wasilewski, A. & Wdowczyk, J. *Proc. 17th int. Cosmic Ray Conf.* **1**, 8-12 (1981).
- Thomson, D. E. *Sci. News* **123**, 405 (1983).

A simple non-markovian equation for a magnetoplasma

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A new kinetic equation for the velocity distribution function f is proposed here, with the innovation that the term $(\partial f / \partial t)_c$ representing the effect of particle collisions, incorporates time delays extending over the interval between successive 90° collisions. When this theory is applied to magnetoplasmas of the type found in tokamaks, it is found that the time delay has a dramatic effect on the heat flux across the magnetic field, giving results orders of magnitude larger than the classical values, and in fair agreement with observations. It appears that non-markovian effects are of great importance in magnetoplasmas, and that they provide answers to some unsolved problems in fusion research.

If $\mathbf{w}$ is the velocity of a cloud of particles at position $\mathbf{r}$, then the collision term in the kinetic equation for the one-particle phase-space density f ,

$$Df \equiv \frac{\partial f}{\partial t} + \mathbf{w} \cdot \frac{\partial f}{\partial \mathbf{r}} + \mathbf{F} \cdot \frac{\partial f}{\partial \mathbf{w}} = \left(\frac{\partial f}{\partial t} \right)_c \quad (\mathbf{F} = \dot{\mathbf{w}}) \quad (1)$$

may depend on the history of f . In general we should assume that it does, but Bogoliubov's¹ hypothesis permits us to ignore time delays in $(\partial f / \partial t)_c$, and while doubts are sometimes expressed, it is usually accepted that the timescale arguments that he devised for neutral gases also apply to fully ionized plasmas.

In a neutral gas the time τ_0 during which particles interact is very much shorter than the time interval τ between successive collisions. This means that the two-particle distribution function implicit in $(\partial f / \partial t)_c$ relaxes to its equilibrium state at a rate τ / τ_0 times more rapidly than f relaxes to its equilibrium state, namely

the maxwellian distribution

$$f_0 = n \left(\frac{m}{2\pi kT} \right)^{3/2} e^{-u^2} \quad \left(\mathbf{u} = \mathbf{c}/C, C^2 = \frac{2kT}{m} \right) \quad (2)$$

Here n is the number density, T is the temperature and $\mathbf{c}$ is the peculiar velocity, that is the difference between $\mathbf{w}$ and its average, $\bar{\mathbf{w}} = \mathbf{v}$.

To take a simple example, the usual relaxation model for $(\partial f/\partial t)_c$ is

$$\left(\frac{\partial f}{\partial t} \right)_c = \tau^{-1} \{ f_0(t) - f(t) \} \quad (3)$$

where f_0 and f are evaluated in the volume element $d\nu = d\mathbf{r} d\mathbf{w}$ of the cloud at the current instant. With this expression, equation (1) is 'markovian', that is, independent of the history of either f_0 or f . But $f_0(t)/\tau$ represents the rate at which particles are collided into $d\nu$ from the background plasma, and we should therefore give f_0 its value just prior to the interaction, at the same time preserving the essential irreversible character of $(\partial f/\partial t)_c$. Let $\text{irr}\{\}$ denote the irreversible part of $\{\}$, that is that part that has positive parity under time reversal; then equation (3) is replaced by

$$\left(\frac{\partial f}{\partial t} \right)_c = \text{irr} \{ f_0(t - \tau_0) - f(t) \} / \tau \quad (4)$$

or

$$\left(\frac{\partial f}{\partial t} \right)_c \approx \tau^{-1} \{ f_0(t) - f(t) \} + \frac{\tau_0^2}{2\tau} D^2 f_0 \quad (5)$$

where the time derivative is evaluated at t , and Df_0 has been omitted in the expansion, since it has the same parity as the left-hand side of equation (1), that is, it is reversible. (Strictly, equation (5) is markovian, since the history has been reduced to a current time derivative, but we shall regard it as non-markovian, to emphasize the distinction between it and equation (3).) With Bogoliubov's hypothesis,

$$\tau_0/\tau \ll 1 \quad (6)$$

equation (5) is reduced to equation (3), and the history of f_0 is thus discarded.

The long range of the Coulomb force throws considerable doubt on equation (6)². A typical charged particle experiences a great number of small-angle collisions, interacting continuously with all of the $n_D = (4\pi/3)\lambda_D^3 n$ particles within a Debye distance, λ_D . The random component of these interactions accumulates over the 'collision' or kinetic time τ to deflect the particle through 90° . In this time the particle typically moves a distance $108\lambda_D n_D$ and hence interacts with $81n_D^2$ particles. Thus in a typical laboratory plasma, a particle experiences $\sim 10^{13}$ grazing interactions per collision interval τ , which means that a 90° 'collision' is essentially a continuous process, lasting for the whole of the kinetic time τ . If we regard the encounter as occurring between a test particle P and the collection Q of particles within the Debye cylinder described above, or better, as a continuous binary encounter between P and a pseudo-particle Q, that lasts for most of the kinetic time τ , then in place of equation (6) we should write

$$\tau_0 = \alpha\tau \quad (\alpha = 0(1)) \quad (7)$$

where α is a constant of order unity.

Provided the expansion

$$f_0(t - \tau_0) = -\tau_0 Df_0 + \frac{1}{2}\tau_0^2 D^2 f_0 \quad (8)$$

remains a good approximation for the somewhat longer τ_0 given by equation (7), equations (1) and (7) yield

$$Df = \tau^{-1} (f_0 - f) + \frac{1}{2}\alpha^2 \tau D^2 f_0 \quad (9)$$

This is our simple, non-markovian kinetic equation. The principle involved here is simply that the equilibrium 'information' available in $d\nu$ is not the instantaneous value $f_0(t)$, but the value

of f_0 about one relaxation time earlier, and the collisional processes tend to drive $f(t)$ irreversibly towards $f_0(t - \alpha\tau)$.

Of course, equation (9) is just a model equation, with a status similar to the relaxation model from which it was derived. But the two alternative non-markovian kinetic theories known to me^{3,4} are so complicated that their consequences for mass and energy transport have not been pursued. At least equation (9) offers the chance of checking the importance of time delays in transport in a plasma, even if there remains some uncertainty in the value of the phenomenological constant α .

In a magnetic field, $\mathbf{B} = B\mathbf{b}$, particles with a charge q have a gyro frequency $\omega_c = qB/m$; we shall be concerned with 'strong' fields, that is, those for which $\omega_c\tau \gg 1$, which means that the cloud of particles in $d\nu$ gyrate about the field many times per 90° collision interval.

Transforming equation (9) from the laboratory frame in which the velocity $\mathbf{w}$ is measured, to a frame moving with the fluid velocity $\mathbf{v}$ and rotating with the local fluid angular velocity, $\boldsymbol{\Omega} = \frac{1}{2}\nabla \times \mathbf{v}$, we find (ref. 5)

$$\mathcal{D}f - \omega_c \mathbf{b} \times \mathbf{c} \cdot \frac{\partial f}{\partial \mathbf{c}} = \tau^{-1} (f_0 - f) + \frac{1}{2}\alpha^2 \tau \mathcal{D}^2 f_0 \quad (10)$$

where

$$\mathcal{D} = D^* + \mathbf{c} \cdot \nabla + (\mathcal{F} - D\mathbf{v} - \mathbf{c} \cdot \mathbf{e}^s) \cdot \frac{\partial}{\partial \mathbf{c}} \quad (11)$$

$\mathbf{e}^s$ is the symmetrical part of $\nabla \mathbf{v}$, $D\mathbf{v}$ is the fluid acceleration, $\mathcal{F} = (q/m)(\mathbf{E} + \mathbf{v} \times \mathbf{B})$, $\mathbf{E}$ is the electric field and for scalars φ , vectors $\mathbf{a}$ and second-order symmetrical tensors $\mathbf{A}$, D^* is defined by

$$\begin{aligned} D^* \varphi &= D\varphi, & D^* \mathbf{a} &= D\mathbf{a} - \boldsymbol{\Omega} \times \mathbf{a}, \\ D^* \mathbf{A} &= D\mathbf{A} - 2\boldsymbol{\Omega} \times \mathbf{A} \end{aligned} \quad (12)$$

with

$$D = \frac{\partial}{\partial t} + \mathbf{v} \cdot \nabla \quad (13)$$

Thus D^* is a frame-indifferent time derivative.

In reducing $D^2 f_0$ to $\mathcal{D}^2 f_0$, we have used equation (2), which gives $\mathbf{b} \times \mathbf{c} \cdot \partial f_0 / \partial \mathbf{c} = 0$, and the fact that $D^2 f_0$ must be evaluated with the coefficients of the derivatives held constant. Note that the rapid gyrations about the field lines have been excluded by changing from D to $\mathcal{D}$, so that now we require that equation (8) holds only for the operator $\mathcal{D}$ and on paths lying roughly parallel to $\mathbf{b}$.

The collision term on the right of equation (10) must conserve the number, momentum and energy of particles, so that

$$\int (1, m\mathbf{c}, \frac{1}{2}m\mathbf{c}^2) \left(\frac{\partial f}{\partial t} \right)_c d\mathbf{c} = 0 \quad (14)$$

where the integral is over the whole of velocity space. To ensure this we are free to add to $(\partial f/\partial t)_c$ a complementary function $(\alpha_0 + \alpha_1 \mathbf{c} + \alpha_2 \mathbf{c}^2)f_0$, where $\alpha_0, \alpha_1, \alpha_2$ are functions of $\mathbf{r}, t$ and independent of $\mathbf{c}$ (ref. 6). We do this later, using equation (14) to determine these parameters.

Let $\varphi = (f - f_0)/f_0$ and assume $\varphi \ll 1$, then from equations (10) and (2)

$$\begin{aligned} \varphi - \omega_c \tau \mathbf{b} \times \mathbf{c} \cdot \frac{\partial \varphi}{\partial \mathbf{c}} &= -\tau \mathcal{D} \ln f_0 + \frac{1}{2}\alpha^2 \tau^2 \{ \mathcal{D}^2 \ln f_0 + (\mathcal{D} \ln f_0)^2 \} \\ (1) & & (2) \end{aligned} \quad (15)$$

where

$$\mathcal{D} \ln f_0 = (u^2 - \frac{5}{2})\mathbf{c} \cdot \nabla \ln T + 2\mathbf{u} \cdot \nabla^0 \mathbf{v} \quad (16)$$

$\nabla^0 \mathbf{v}$ being the traceless part of $\mathbf{e}^s$. We shall assume ∇T to be parallel to unit vector $\hat{\mathbf{r}}$ and perpendicular to $\mathbf{b}$. With $\omega_c\tau$ very large the term (1) in equation (15) yields a heat flux vector parallel to ∇T of the standard type⁷,

$$\hat{\mathbf{r}} \cdot \mathbf{q}^{(1)} = -K_\perp \hat{\mathbf{r}} \cdot \nabla T \quad (K_\perp \sim K_\parallel / (\omega_c\tau)^2) \quad (17)$$

where $K_{\parallel}$ is the thermal conductivity parallel to $\mathbf{b}$. On the other hand it is easily deduced from equation (16) (details will be published elsewhere) that the non-markovian term (2) in equation (15) gives

$$\hat{\mathbf{r}} \cdot \mathbf{q}^{(2)} = 7\alpha^2 \tau \frac{kp}{qB} H \hat{\mathbf{r}} \cdot \nabla T \quad (H = \mathbf{b} \times \hat{\mathbf{r}} \cdot \nabla \mathbf{v} \cdot \hat{\mathbf{r}}) \quad (18)$$

The choice $\alpha = (2.5/7)^{1/2} \approx 0.6$ for the electron gas gives

$$\hat{\mathbf{r}} \cdot \mathbf{q}_e^{(2)} = -K_e^* \hat{\mathbf{r}} \cdot \nabla T_e \quad \left(K_e^* \equiv \frac{5kp_e}{2eB} \tau_e H_e \right) \quad (19)$$

a result previously obtained⁸ by quite different arguments. It was shown that in laboratory plasmas K_e^* is several hundred times larger than $K_{\perp e}$. Thus non-markovian effects are of great importance in the transport theory of a magnetoplasma. They may account for the relative failure of tokamaks and similar magnetoplasma machines to confine the plasma energy in the manner originally optimistically predicted by the standard theory. Provided τ_e in equation (19) is replaced by an average bounce time between the tokamak mirrors (good physical arguments can be advanced for this substitution), comparison of equation (19) with observations in tokamaks shows remarkably good agreement. Another interesting feature of equation (19) is that at sufficiently high densities H_e becomes negative and a thermal instability results that must disrupt the discharge; this may explain the well known disruptive instability in tokamaks⁹.

To pursue these technical topics here would be inappropriate. The conclusion is that non-markovian effects are of great importance in a magnetoplasma, and that they appear to provide the answers to several unsolved problems in fusion research.

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1. Bogoliubov, N. N. *Studies in Statistical Mechanics* Vol. 1 (eds Boer, J. De & Uhlenbeck, G. E.) (North-Holland, Amsterdam, 1946).
2. Montgomery, D. *Lectures in Theoretical Physics* Vol. 9C (ed. Brittin, W. E.) (Gordon and Breach, New York, 1967).
3. Tschen, C. M. *Phys. Rev.* **114**, 394–411 (1959).
4. Fujita, S. *Lectures in Theoretical Physics* Vol. 9C (ed. Brittin, W. E.) (Gordon and Breach, New York, 1967).
5. Woods, L. C. *J. Fluid Mech.* **136**, 423–433 (1983).
6. Chapman, S. & Cowling, T. G. *The Mathematical Theory of Non-Uniform Gases* (Cambridge University Press, 1970).
7. Bragninskii, S. I. *Reviews of Plasma Physics* Vol. 1 (ed. Leontovich, M. A.) (Consultants Bureau, New York, 1965).
8. Woods, L. C. *J. Plasma Phys.* **29**, 143–154 (1983).
9. Murakami, M., Callen, J. D. & Berry, L. A. *Nucl. Fus.* **16**, 347–354 (1976).

A possible mechanism for epeirogenic uplift

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Continental geology is dominated by vertical movements, some of these are the result of crustal shortening and extension associated with large horizontal displacements, involving processes which are now understood, at least in outline. However, both uplift and subsidence can occur without associated shortening or extension of the continental crust. Most investigations of such epeirogenic movements have been concerned with subsidence, partly because of the economic importance of the resulting sedimentary basins, and partly because of the existence of a simple model for the process¹ which can account for the development of several basins in some detail^{2–4}. However, uplift has been relatively neglected and cannot be produced by the same mechanism. The purpose of this paper is to examine whether epeirogenic uplift can result from the intrusion of large thicknesses of basic magma into the lower part of continental crust. This suggestion was considered as a possible mechanism for the regional uplift of the Colorado Plateau by Gilbert⁵ more than 100 years ago, and also by Holmes⁶ (though

both rejected the idea). However, a recent discussion of no less than 14 different mechanisms for generating uplift⁷ did not include this process, even though it seems able to produce the observed vertical motions and also to account for some of the features of the evolution of sedimentary basins that cannot be explained by the uniform stretching model^{3,8,9}. Although the suggestion is not new, the argument used here to support it is, and depends on an understanding of the relationship between ocean island volcanoes and mantle circulation.

In the ocean basins magma is generated in two different environments. Plate separation at ridges leads to adiabatic upwelling and melting. This process produces most of the oceanic crust. The other type of feature which produces magma in the ocean basins is intra-plate volcanism such as that which is occurring in Hawaii, Iceland and the Azores. No detailed estimates of the volume of volcanic material produced by intra-plate volcanism appear to have been made. Ben-Avraham *et al.*¹⁰ suggest that about 10% of the ocean floor is covered by aseismic plateaux, almost all of which are volcanic constructs. The crustal thickness beneath these features is about 20 km, of which perhaps 15 km has been added to the normal oceanic crustal thickness by volcanism. The error in the estimate of the total volume involved may be as much as a factor of two; more accurate estimates are not critical to the present argument. Many of the major ocean island volcanoes which are now active are believed to be at the centre of hot rising regions within the mantle^{11–13}. Although the nature of the relationship between the magma and the upwelling is not yet clear, it is generally agreed to exist. The importance of this association is that hot rising regions are generated at the base of the convecting region¹¹ by instabilities of the lower thermal boundary layer. The distribution of these regions, and hence the rate of magma production from such sources, should therefore be independent of the distribution of continental and oceanic lithosphere, though they cannot yet be mapped beneath the continents by the methods used in the oceans. Many authors have proposed continental volcanoes which they believe to be the surface expression of such rising regions. Certainly some of these volcanoes are likely to have such an origin. There is a major difficulty to be overcome, however, if the rate of magma production in the rising regions is to be the same beneath both continents and oceans. The mean age of oceanic lithosphere is no more than 100 Myr, whereas that of the continental lithosphere is probably at least 1,500 Myr. Hence 15 times more of the continent than of the ocean should be covered by a basaltic layer 15 km thick. Such basalt should therefore make up about 75% of the continental crust of 30 km thickness. Compared with this estimate the volume of flood basalts even in an area such as the Deccan is negligible. Clearly this argument is not compatible with geological observations if the magma is erupted at the surface.

The geological observations are, however, not contradicted if this volume of magma is injected into the lower half of the continental crust. The mean density of the continental crust is about 2.8 Mg m⁻³ (ref. 14), and varies from about 2.6 Mg m⁻³ at the top to 3 Mg m⁻³ at the bottom. The composition of the intruding magma has little effect on its density, which is likely to be between 2.7 and 2.9 Mg m⁻³ (ref. 15) at a depth of 30 km. If much of the primary magma is picritic, as seems likely^{16,17}, its density will be at the upper end of this range. Therefore, whatever its composition, such a magma could form sills within the plate. This emplacement is likely to be within the crust if the magma density is less than that of the lower crust, or it could form a layer between the crust and the mantle if its density is greater. If the whole of the lower part of the continental crust is produced in this way, the resulting velocity of P waves should be between 6.8 and 7.0 km s⁻¹, in agreement with that observed. Cox¹⁷ has proposed a similar model on petrological grounds, and argued that differentiation within a picritic layer could generate plateau basalts. Large intrusions of basic magma could also provide the source of heat required to generate rhyolites and granites from the continental crust by anatexis^{18–20}, and in this way satisfy the isotope constraints^{21–23}. Such intrusions into

the lower crust could account for the horizontal layering seen in many deep reflection profiles of the continental crust (refs 24, 25 and D. H. Matthews, personal communication). Clear reflections from the Moho also suggest that extensive intrusion of dykes is not common. The resulting uplift, followed by erosion, can explain the dominance of high grade metamorphic rocks in the surface outcrop of Archaean terrains²⁶.

Perhaps the most important consequence of the intrusion of great thicknesses of magma into the lower crust is the associated surface uplift. The addition of 15 km of material of density 2.7 Mg m^{-3} to the crust which overlies mantle of density 3.3 Mg m^{-3} produces 2.7 km of permanent uplift, sufficient to generate features like the Colorado Plateau. The reason Gilbert⁵ argued against such a mechanism is that the plateau is bounded by linear monoclines, whereas the smaller intrusions he mapped in the Henry Mountains were circular in plan. However, it would not be surprising if the uplift reactivated old lines of weakness in the upper crust as faults that may underlie the monoclines. Gilbert's objection does not therefore seem to be fundamental. Some uplifted regions, such as Lesotho in southern Africa²⁷, have probably existed for longer than 60 Myr, the thermal time constant of the lithosphere. This region is also more than 100 km from the continental margin. Such uplift is therefore unlikely to be produced by lithospheric heating²⁸, which would not extend more than about 100 km from the margin and would decay with a time constant of 60 Myr. There is, however, no difficulty in producing the uplift by large intrusions, and the resulting elevation can only be removed by erosion. Furthermore, many uplifted regions are associated with extensive surface volcanism.

These arguments therefore suggest that large igneous intrusions are a major cause of uplift. Another cause is likely to be the uplift produced by the hot upwelling mantle. In oceanic regions such uplift has a scale of 1,000 km or more, and an amplitude of about 1 km. Similar surface deformation should exist in continental regions, although the subaerial amplitude will be reduced to about 700 m. Such features should be widespread, though their existence has not been clearly established. Unlike uplift produced by crustal thickening, that resulting from mantle upwelling will disappear as the plate moves away from the rising region. Also, the horizontal scale of the uplift produced by mantle upwelling should be greater than that from igneous intrusion. The source of the magma may be the hot upwelling region, especially in regions remote from plate boundaries. This is the only source whose production rate can be estimated. Near plate boundaries basic magma is generated in other ways, by both extension and subduction, and may also be emplaced into continental crust.

The above discussion has been concerned with uplift which is not directly associated with crustal shortening or extension. It is likely that some major igneous intrusions into the lower crust also occur in continental regions undergoing deformation. If such additions occur during the extension associated with the formation of a sedimentary basin, the volume of the crust will not be conserved. This process has been discussed by several authors^{9,29}, all of whom required that there be a relationship between the extension and the amount of melt produced. No such relationship is, in general, to be expected unless both the temperature and composition of the mantle are constant. The ratio of the final to the initial area, β , cannot then be estimated from the change in crustal thickness. Since the density of the intrusion is similar to the mean crustal density ρ_c , the initial tectonic subsidence, s_i , is approximately related to the initial, t_c , and final, t_f , crustal thicknesses by

$$s_i = \frac{(\rho_m - \rho_c)(t_c - t_f)}{(\rho_m - \rho_w)} \quad (1)$$

where $t_c > t_f$, ρ_m is the density of the mantle, ρ_w that of seawater and the upper surface of the crust is initially at or below sea level. The final thickness t_f then consists of the sum of the

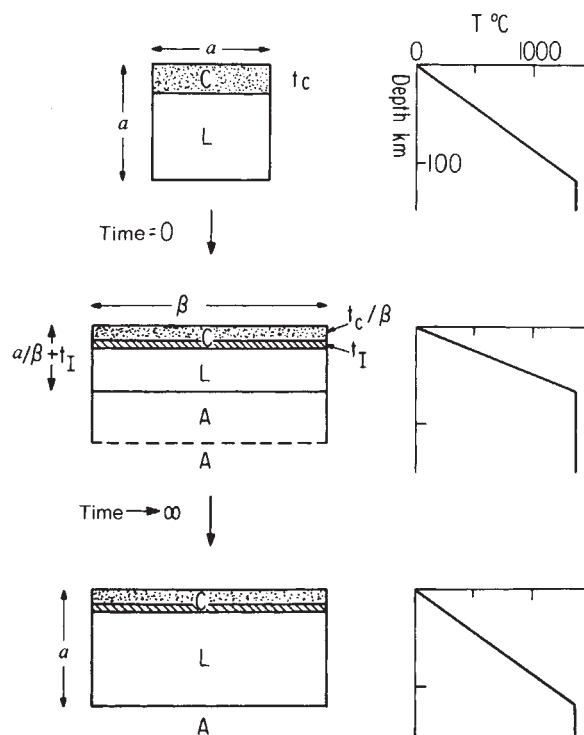


Fig. 1 A piece of thermally equilibrated continental lithosphere of thickness a , consisting of crust C (dotted) and mantle L , underlain by asthenosphere A , is uniformly extended by a factor β at time 0, and a thickness t_i of basic magma (cross-hatched) is added to the crust. The total change in thickness of the continental crust, marked C , is $t_c(1 - 1/\beta) - t_i$ and controls the tectonic subsidence associated with faulting during the stretching event. In contrast, the variation of temperature with depth is controlled by β , as are also the heat flux and the thermal subsidence. In the example illustrated $\beta = 2$ and $t_i = 7.5$ km. Hence the value of β obtained from the change in crustal thickness would be 1.33.

thickness t_c/β of original crust and of that, t_i , of intrusions (Fig. 1). The igneous material will probably not be emplaced as dykes but as sills, because the density of most magma types exceeds that of upper crustal material. After the extension ceases, the thermal subsidence and heat flux will be controlled by the extension of the lithosphere, which will exceed that (β_a) estimated from the change in crustal thickness. The heat flux associated with the intrusion itself will decay quickly, because the thermal time constant of a 30 km thick layer is less than 5 Myr.

The effects of extension combined with large additions to the crust therefore closely resemble those which Royden and Keen⁸ and Royden *et al.*³ have argued should be produced by stretching the mantle part of the lithosphere more than the crust. The behaviour of the model proposed above is the same as that of Royden *et al.* if their crustal value of β , β_a , is defined to be

$$\beta_a = t_c/(t_i + t_c/\beta) \quad (2)$$

The value of β_a controls the change in crustal thickness and initial subsidence, whereas the true value of β controls the heat flux and thermal subsidence. In regions such as the Pannonian Basin the magma is more likely to be produced by the subduction zone than by the hot rising jet within the mantle.

Despite the similarity in the behaviour of the model proposed here to that of Royden *et al.*³, the physical processes involved are rather different. If the crust is everywhere stretched less than the mantle part of the lithosphere, as they suggest, a space problem arises. If, however, the true crustal extension is the same as that of the mantle, but merely appears to be less because crustal volume has not been conserved, there is no such difficulty. In the Pannonian Basin the thermal subsidence requires a value

of β of at least 3, whereas the present crustal thickness and the initial subsidence suggest a value of β of about 2. If the initial crustal thickness was 36 km, these values require the addition of 6 km or more of igneous material. Such a thickness is not unreasonable, as large amounts of volcanic material were being erupted in the surrounding regions during the extensional event, probably generated by a subduction zone. Whether the large horizontal displacements, which must occur if β is to have a value of more than 3, can be accommodated by faulting is uncertain. Though this objection has been raised repeatedly^{30,31} against the stretching model even in the absence of additions

to the crust, the accuracy of estimates made from fault geometry is still controversial³². In contrast to the Pannonian Basin, little volcanism was associated with the extension of the North Sea, and therefore different methods of estimating β yield similar values⁴.

Massive intrusions can also occur in regions undergoing shortening, where they will add to the uplift produced by crustal thickening.

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- McKenzie, D. P. *Earth planet. Sci. Lett.* **40**, 25–32 (1978).
- Slater, J. G. & Christie, P. A. F. *J. geophys. Res.* **85**, 3711–3739 (1980).
- Royden, L., Horvath, F., Nagymarosy, A. & Stegena, L. *Tectonics* **2**, 91–137 (1983).
- Wood, R. J. & Barton, P. *Nature* **302**, 134–136 (1983).
- Gilbert, G. K. *Report on the Geology of the Henry Mountains* (Government Printing Office, Washington DC, 1877).
- Holmes, A. *Principles of Physical Geology* (Ronald, New York, 1964).
- McGetchin, T. R., Burke, K. C., Thompson, G. A. & Young, R. A. in *Dynamics of Plate Interiors* (eds Bally, A. W., Bender, P. L., McGetchin, T. R. & Walcott, R. I.) 99–110 (Am. Geophys. Un. & Geol. Soc. Am., Boulder, Colorado, 1980).
- Royden, L. & Keen, C. E. *Earth planet. Sci. Lett.* **51**, 343–361 (1980).
- Beaumont, C., Keen, C. E. & Boutilier, R. *Geophys. J. R. astr. Soc.* **70**, 667–715 (1982).
- Ben-Avraham, A., Nur, A., Jones, D. & Cox, A. *Science* **213**, 47 (1981).
- McKenzie, D. P., Roberts, J. M. & Weiss, N. O. *J. Fluid Mech.* **62**, 465–538 (1974).
- Slater, J. G., Lawver, L. A. & Parsons, B. *J. geophys. Res.* **80**, 1031–1052 (1975).
- Watts, A. B. *J. geophys. Res.* **81**, 1533–1553 (1976).
- Cochran, J. R. *Geophys. J. R. astr. Soc.* **68**, 171–201 (1982).
- Nisbet, E. G. in *Komatiites* (eds Arndt, N. T. & Nisbet, E. G.) Ch. 29 (Allen & Unwin, London, 1982).
- O'Hara, M. J. *Earth Sci. Rev.* **4**, 69–133 (1968).
- Cox, K. G. *J. Petrol.* **21**, 629–650 (1980).
- Pichler, H. & Ziel, W. *Bull. Volcan.* **35**, 424–452 (1972).
- Wyllie, P. J. *Tectonophysics* **43**, 41–71 (1977).
- Ewart, A. *J. Petrol.* **23**, 344–382 (1982).
- Ewart, A. & Stipp, J. J. *Geochim. cosmochim. Acta* **32**, 699–736 (1968).
- Klerkx, J., Deutsch, S., Pichler, H. & Ziel, W. *J. Volcan. geotherm. Res.* **2**, 48–71 (1977).
- Mahoney, J. et al. *Earth planet. Sci. Lett.* **60**, 47–60 (1982).
- Smythe, D. K. et al. *Nature* **299**, 338–340 (1982).
- Oliver, J., Cook, F. & Brown, L. *J. geophys. Res.* **88**, 3329–3347 (1983).
- Perkins, D. & Newton, R. C. *Nature* **292**, 144–146 (1981).
- King, L. *The Morphology of the Earth* (Oliver & Boyd, Edinburgh, 1967).
- Sleep, N. H. *Geophys. J. R. astr. Soc.* **24**, 325 (1971).
- Foucher, J.-P., Le Pichon, X. & Sibuet, J.-C. *Phil. Trans. R. Soc. A305*, 27 (1982).
- Ziegler, P. A. *Phil. Trans. R. Soc. A305*, 113–143 (1982).
- Avedik, F. et al. *Phil. Trans. R. Soc. A305*, 5 (1982).
- Bally, A. W. *Phil. Trans. R. Soc. A305*, 41 (1982).

Isotopic and geochemical evidence for the evolution of a cyclic unit in the Rhum intrusion, north-west Scotland

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Layered basic intrusions can record processes which operated within basic magma chambers. In particular processes such as magma mixing and fractionation can be identified from the chemistry of cumulus minerals. Isotopes have been used to identify contamination in lavas^{1–6}, but they can also be applied to cumulates to infer a continuous record of the interactions of crust and magma within the magma chamber. We report here that olivine compositions, rare earth elements (REE) on mineral separates and Sr isotopes on whole rocks and pyroxenes from unit 9 of the Eastern Layered Series of the Tertiary Rhum intrusion, north-west Scotland, suggest that fresh magma entered the chamber and mixed with fractionated and contaminated magmas at the base of the chamber. With prolonged residence in the chamber the fresh magma became fractionated and contaminated, was then erupted off and replaced by fresh magma which produced the basal peridotites of unit 10.

The Eastern Layered Series (ELS) comprises 15 cyclic units⁷, each representing the crystallized products of successive injections of magma into the chamber^{7,8}. A typical sequence of rocks within a cyclic unit comprises peridotite, grading upwards into troctolite and finally gabbro, corresponding to the first appearance of olivine, plagioclase and the clinopyroxene on the liquidus with decreasing temperature. The basal peridotites are in sharp contact with the gabbros of the preceding unit.

Unit 9 is 21 m thick (Fig. 1), olivine being the only cumulus mineral present throughout the unit. The forsterite content can be directly related to the Mg/Mg+Fe(II) ratio of the magma (an index of fractionation)⁹.

The most Mg-rich olivines would be expected at the base and the most Fe-rich olivines at the top of the unit. In unit 9 the

olivines are more Mg rich upsequence through the peridotites (Fig. 2, Table 1). Within the troctolites the compositions vary, while the olivines within the gabbros become more Fe-rich, particularly towards the top of the unit. The base of unit 10 has more Mg-rich olivines than the top of unit 9. This would, perhaps, be consistent with the eruption of fractionated magma and replacement by fresh magma.

In contrast, the olivines in the gabbros at the top of unit 8 have almost identical compositions with those in the basal peridotite of unit 9, while olivines 2 m below the unit 8/9 contact (41597) are the most evolved yet found in the ELS. The basal peridotite (42142) of unit 9 contains a unique occurrence of cumulus clinopyroxene, restricted to the basal metre of unit 9. They form up to 15% of the mode and are small subhedral crystals occasionally enclosed within cumulus olivine, but more typically within intercumulus pyroxene and plagioclase. They are unzoned with compositions comparable with the cumulus pyroxenes in the gabbros at the top of unit 8.

The approximate equivalence of olivine and pyroxene compositions across unit contacts (Table 1) and the progressively more primitive nature of olivines upsequence through the peridotites has been interpreted as being due to magma mixing¹⁰ between a fractionated magma and a fresh magma batch. The presence of cumulus pyroxenes at the base of unit 9 may support this.

Sr isotopes measured on whole rocks and pyroxene separates are presented in Fig. 2 and Table 1. Rb/Sr measurements on several samples indicate that the age correction (60 Myr) is insignificant and within experimental error, therefore ratios where no Rb data are available are assumed to be initial ratios. The variations in ⁸⁷Sr/⁸⁶Sr through unit 9 are large.

Clinopyroxenes through unit 9 are generally in isotopic equilibrium with the whole rocks. The only exceptions are at the tops of units 8 and 9. Disequilibrium between pyroxene and magma can be caused by magma mixing between two or more isotopically distinct magmas with the pyroxenes being derived from one magma¹¹. Because the rate of Sr diffusion in pyroxene is slow compared with that in the magma¹², it is possible for the pyroxene to retain the ⁸⁷Sr/⁸⁶Sr of the magmas it originally crystallized from and not reequilibrate with the magma it settled in. Pyroxenes are also resistant to hydrothermal alteration¹³. Because the pyroxenes were leached¹⁴, any hydrothermal Sr will be removed, therefore because pyroxenes and whole rock ⁸⁷Sr/⁸⁶Sr are equivalent these variations are considered primary and not the result of hydrothermal alteration.

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Table 1 Composition analyses

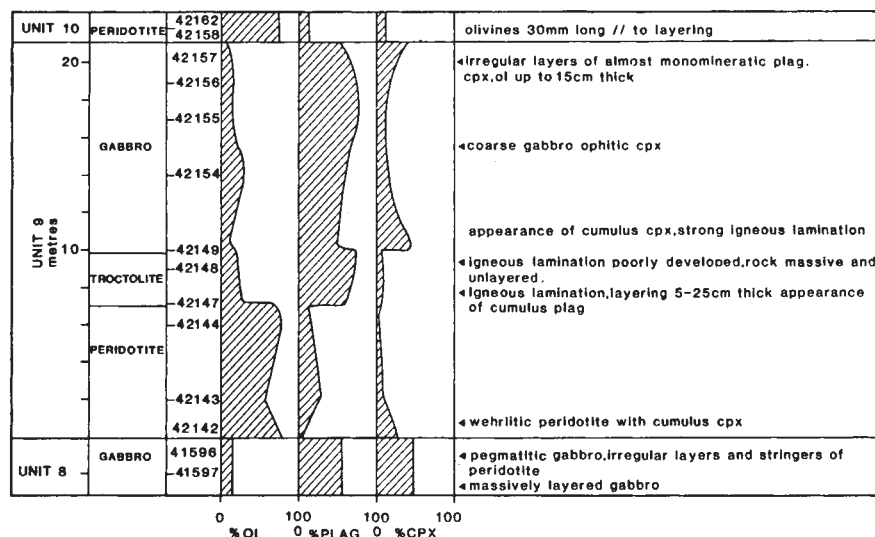
Sample		Unit (height above base, m)	Rb‡ (p.p.m.)	Sr‡ (p.p.m.)	Rb/Sr	$^{87}\text{Sr}/^{86}\text{Sr}^\dagger$	$^{87}\text{Sr}/^{86}\text{Sr}_{60}^\ddagger$	Fe mole % olivine	Mgr CPX
42162 Peridotite	WR	10(1)	ND	69	—	0.70411 ± 5	—	85.2 ± 0.2	—
42158 Peridotite	WR	10(0)	0.25	56.1	0.004	0.70382 ± 7	0.70381	86.3 ± 0.4	—
42157 Gabbro	WR	9(21)	0.92	265.8	0.003	0.70538 ± 6	0.70537	76.6 ± 0.1	—
	CPX		ND	24.9	—	0.70495 ± 12	—	—	—
42156 Gabbro	WR	9(19)	ND	307.9	—	0.70425 ± 5	—	79.2 ± 0.1	—
42155 Gabbro	WR	9(17)	1.55	311.6	0.005	0.70395 ± 4	0.70394	79.9 ± 0.7	—
42154 Gabbro	WR	9(14)	1.35	238.7	0.005	0.70405 ± 3	0.70404	78.9 ± 0.3	—
	CPX		ND	ND	—	0.70397 ± 7	—	—	—
42149 Gabbro	WR	9(10)	0.87	286.1	0.003	0.70413 ± 3	0.70412	80.1	—
	CPX		ND	28.36	—	0.70417 ± 4	—	—	—
42148 Troctolite	WR	9(9)	ND	314.7	—	0.70550 ± 7	—	84.2 ± 0.2	—
42147 Troctolite	WR	9(7)	0.43	204.6	0.002	0.70496 ± 5	0.70496	82.6 ± 0.3	—
42144 Peridotite	WR	9(6)	0.55	125.9	0.004	0.70544 ± 6	0.70543	84.4 ± 0.04	—
	CPX		ND	30.29	—	0.70543 ± 4	—	—	—
42143 Peridotite	WR	9(2)	0.55	141.7	0.004	0.70532 ± 5	0.70531	83.6 ± 0.2	—
42142 Peridotite	WR	9(0)	0.44	138.5	0.003	0.70525 ± 2	0.70525	81.5 ± 0.5	83 ± 0.6
	CPX		ND	28.3	—	$0.70530 \pm$	—	—	—
41596 Gabbro	WR	8 (top)	ND	275.6	—	0.70622 ± 2	—	81.1 ± 0.2	83.8 ± 0.17
	CPX		0.14	50.4	0.0027	0.70633 ± 5	0.70633	—	—
41597 Gabbro	WR	8 (2m below top)	ND	289.5	—	0.70543 ± 2	—	73.8 ± 0.4	—

WR, whole rock; CPX, clinopyroxene.

* Isotope dilution (p.p.m.).

† Errors 2 s.e. SRM 987 0.710254 ± 12 (47 analyses); VG-MM 30. Mgr, $\text{Mg}/(\text{Mg} + \text{Fe(II)})$, 10 analyses errors 1 s.e. ND, not determined.‡ 60 Myr; $\lambda 1.42 \times 10^{-11}$.

§ Mean at least five analyses by WDS. Errors 1 s.e.

Fig. 1 Lithological log through unit 9.

The unit can be divided isotopically into two. In the peridotites $^{87}\text{Sr}/^{86}\text{Sr}$ is ~ 0.705 , while the gabbros are mainly ~ 0.704 . The troctolites appear to have transitional values. Within the gabbros an increase in $^{87}\text{Sr}/^{86}\text{Sr}$ at the top of unit 9 coincides with Fe enrichment in olivine and may represent the process of combined assimilation of high $^{87}\text{Sr}/^{86}\text{Sr}$ crust and fractional crystallization (AFC)³. A sharp reduction in $^{87}\text{Sr}/^{86}\text{Sr}$ at the base of unit 10 coincides with more Mg olivines which may suggest that contaminated and fractionated magmas were erupted off and replaced by relatively uncontaminated and unfractionated magmas.

In the peridotites of unit 9 a more complex situation is present. The olivine compositions indicate that magma mixing has operated. The Sr isotopes may support this because the $^{87}\text{Sr}/^{86}\text{Sr}$ of the peridotites is substantially higher than the basal peridotites of unit 10 and may represent a mixture of high $^{87}\text{Sr}/^{86}\text{Sr}$ magma which crystallized the top gabbro of unit 8 and low $^{87}\text{Sr}/^{86}\text{Sr}$ fresh magma.

The REE contents of separated pyroxenes are presented in

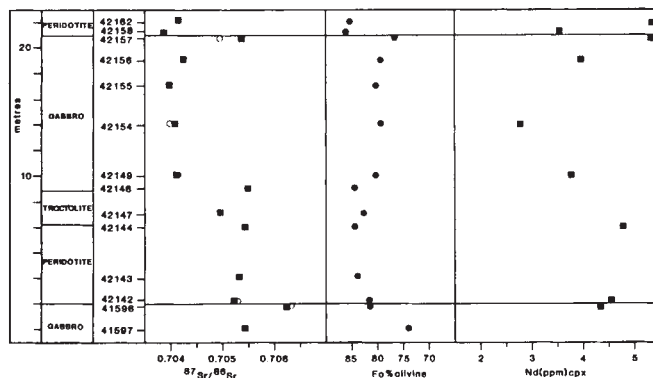
**Fig. 2** Variation in whole rock (squares) and clinopyroxene (circles) $^{87}\text{Sr}/^{86}\text{Sr}$ through unit 9, compared with Fe content in olivine and Nd concentration in clinopyroxene.

Table 2 REE by isotope dilution

Sample	La	Ce	Nd	Sm	Eu	Gd	Dy	Er	Yb
42162 CPX*	0.90	3.60	5.33	2.00	0.69	2.60	2.75	1.37	1.00
42158 CPX	0.59	2.66	3.57	1.36	0.47	1.79	1.78	0.90	0.68
42157 CPX	0.83	3.73	5.34	2.15	0.75	2.92	2.99	1.54	1.20
42156 CPX	—	2.94	3.99	1.54	0.48	—	—	—	0.81
42154 CPX	0.44	2.06	2.77	1.08	0.38	1.42	1.45	0.71	0.55
42149 CPX	0.79	2.77	3.790	1.48	0.49	1.95	—	0.94	0.83
42144 CPX	0.79	3.63	4.79	1.83	0.58	2.42	—	1.29	1.000
42142 CPX	—	3.78	4.57	1.75	0.56	2.29	—	1.29	0.97
41596 CPX	0.77	3.26	4.360	1.65	0.52	2.03	—	1.19	0.94

From ref. 15. Errors 2 s.e. are 1%. CPX, clinopyroxene.

Table 2. The REE patterns are parallel, with variable, small, negative Eu anomalies. Because the patterns are parallel the total REE content (Σ REE) of the pyroxenes can be expressed in terms of one element, Nd, for example. The variation of Nd through unit 9 (Fig. 2) is remarkably similar to $^{87}\text{Sr}/^{86}\text{Sr}$. Although Nd can increase in magma during fractionation, $^{87}\text{Sr}/^{86}\text{Sr}$ cannot, consequently, these variations represent the addition of a high Nd, high $^{87}\text{Sr}/^{86}\text{Sr}$ contaminant. This addition is seen in the gabbros at the top of unit 9 and coincides with

fractionation. The peridotites of unit 9 also seem to be contaminated. However, this contamination could be the result of magma mixing between the fresh magma influx and any contaminated magma left in the chamber.

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- DePaolo, D. & Wasserburg, G. *Geochim. cosmochim. Acta* **43**, 615–627 (1979).
- James, D. E. *Earth planet. Sci. Lett.* **57**, 47–62 (1982).
- DePaolo, D. *Earth planet. Sci. Lett.* **53**, 189–202 (1982).
- Moorbath, S. & Welke, H. *Earth planet. Sci. Lett.* **5**, 217–230 (1969).
- Moorbath, S. & Thompson, R. N. *J. Petrol.* **21**, 295–321 (1980).
- Dickin, A. P. *J. Petrol.* **22**, 155–189 (1980).
- Brown, G. M. *Phil. Trans. R. Soc. B240*, 1–53 (1956).

- Huppert, H. E. & Sparks, R. S. J. *Contr. Miner. Petrol.* **75**, 279–289 (1980).
- Roeder, P. L. & Emslie, R. F. *Contr. Miner. Petrol.* **29**, 275–289 (1970).
- Dunham, A. C. & Wadsworth, W. J. *Min. Mag.* **42**, 347–356 (1979).
- Vollmer, R., et al. *Geotherm. Res.* **11**, 317–327 (1981).
- Sneeringer, M. & Hart, S. *EOS* **59**, 402 (1978).
- Taylor, H. P. & Forester, R. W. *J. Petrol.* **20**, 355–419 (1979).
- Stosch, H. G., Carlson, R. W. & Lugmair, G. W. *Earth planet. Sci. Lett.* **47**, 263–271 (1980).
- Thirlwall, M. F., *Chem. Geol.* **35**, 155–166 (1982).

Oxygen isotope calibration of the onset of ice-rafting and history of glaciation in the North Atlantic region

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We report here that DSDP Site 552A, cored with the hydraulic piston corer on the west flank of Rockall Bank, recovered an undisturbed sequence of alternating white deep-sea carbonate oozes and dark-coloured layers that are rich in glacial debris. Oxygen isotope analysis of the sequence together with detailed nannofossil and palaeomagnetic stratigraphy shows that the first major horizon of ice-rafting occurred at about 2.4 Myr, and was preceded by a minor pulse of ice-rafting at about 2.5 Myr. The carbon isotope record shows that the site has been bathed by a water mass of similar characteristics to present-day North Atlantic deep water at least since 3.5 Myr.

Despite the concentration of members of the deep-sea drilling community around the North Atlantic, surprisingly few Deep Sea Drilling Project sites have been drilled that are suitable for even a cursory study of North Atlantic palaeoenvironments. Until recently the best sites in which to study the onset of glaciation around the North Atlantic were from DSDP Leg 12, sites 111 and 116, in which Berggren¹ studied the stratigraphic

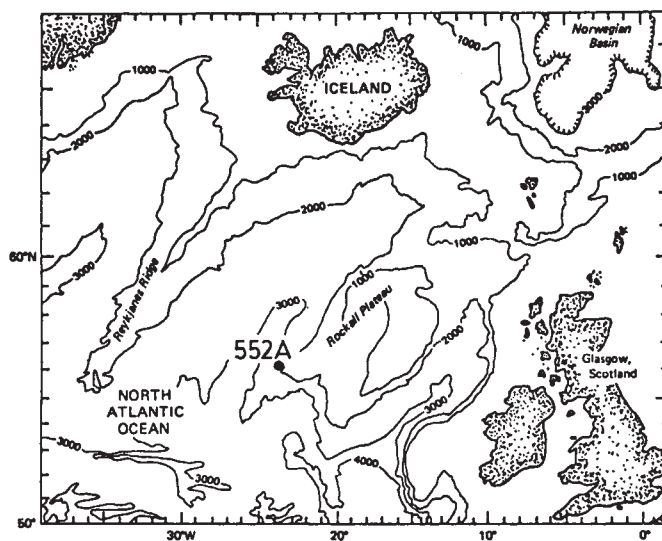


Fig. 1 Location map for Site 552A (56°02.56' N, 23°13.38' W, 2,311 m water depth).

position of the earliest ice-rafted debris in the region and estimated its age at 3.0 Myr (Backman² re-examined this material and obtained a younger estimate of 2.5 Myr). Sediments from the Leg 12 sites were extensively disturbed by rotary drilling, and not suitable for detailed analysis of the palaeoenvironmental record. During Leg 81, the *Glomar Challenger* used the newly developed hydraulic piston corer (HPC)³ with considerable success, and in particular recovered a section from the west flank of Rockall Bank (Fig. 1) that is largely undisturbed.

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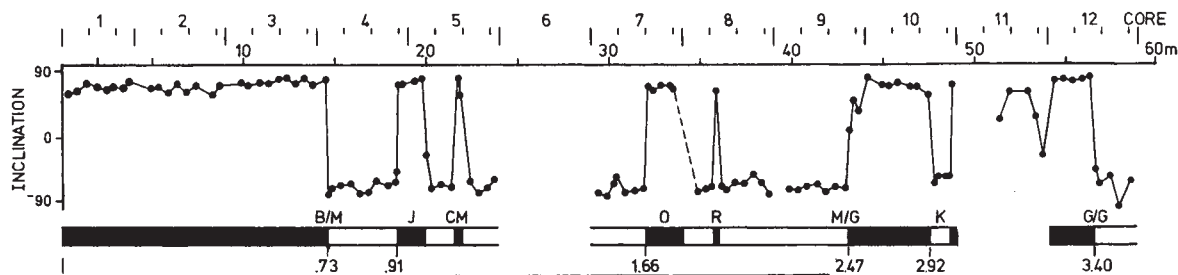


Fig. 2 Magnetic record for Site 552A. Demagnetized inclinations are shown only for apparently undisturbed parts of the cores (the data from core 11 suggest that some unrecognized disturbance may be present).

The upper 40 m of the section comprises alternating units of white and dark grey colour. In the white intervals, the $>150\text{-}\mu\text{m}$ fraction consists almost exclusively of foraminifera; in the dark intervals angular sand-sized rock fragments predominate although sufficient benthic foraminifera for isotopic analysis are present at almost every horizon examined. The dark-coloured intervals containing ice-rafted material are visually clear and also show unmistakably in a record of carbonate content (typically between 80 and 90% in the white layers and 10–40% in the dark layers).

We have made isotope analyses at 10-cm intervals over the interval from well below the first ice-rafting layer, through to the recent sediment. Only core 6 was excluded from analysis because it was very severely disturbed by coring. Three species were used: *Globocassidulina subglobosa*, *Uvigerina peregrina* and *Planulina wuellerstorfi*. In Figs 3 and 4 data are presented using the previously established 'correction factors' to relate the oxygen isotope values obtained for *G. subglobosa* and *P. wuellerstorfi* to the equilibrium value which is thought to be given by *Uvigerina* spp.^{4,5}. Carbon isotope values are related to *P. wuellerstorfi* rather than to *Uvigerina* because this procedure provides a good estimate of the ^{13}C content of ocean dissolved CO_2 (refs 6, 7).

Samples for palaeomagnetic analysis were taken approximately every 25 cm, avoiding sediment that showed any sign of disturbance. Only orientation with respect to the vertical axis was preserved so that the inclination component is used to infer polarity at this high-latitude site. The natural remanent magnetization (NRM) was measured on a two-axis cryogenic magnetometer⁸ both before and after alternating-field demagnetization at 10–30 mT. NRM intensities are typically of the order 10^{-2} A m^{-1} in the upper 45 m of the section, decreasing to about 10^{-3} A m^{-1} in the lower part. Measured inclination data are shown in Fig. 2. Inclinations are generally well-grouped near to the expected dipole value (71° , positive for normal and negative for reversed) for the latitude of the site, indicating that a reliable record of the geomagnetic field has been obtained to the base of the Gauss normal magnetochron. The lower boundary of the Olduvai subchron is unfortunately obscured by disturbance at the break between successive cores, while the Mammoth subchron is unclear, probably lying in a slightly disturbed section of core 11.

Analyses of the carbonate content of the sediment have also been made at 10-cm intervals through the upper 59 m of the section. In this part of the North Atlantic, carbonate content is controlled chiefly by variations in the influx of non-carbonate material transported by ice⁹. Changing carbonate production also has a major role, while carbonate dissolution has little impact at such shallow water depths in the North Atlantic.

The samples taken for stable isotope analysis were also used for quantitative nannofossil studies; here we show (Fig. 4) only those nannofossil data which have a bearing on the biostratigraphical position of the early ice-rafting horizon.

Figure 3 shows the oxygen isotope and carbonate data for the upper five cores, together with the oxygen isotope record of core V28-238 (ref. 4) for comparison. Both records are plotted linearly with respect to depth in sediment between magnetic reversals. Since it has been established that the sediment in V28-238 accumulated at a fairly regular rate¹⁰, the axis

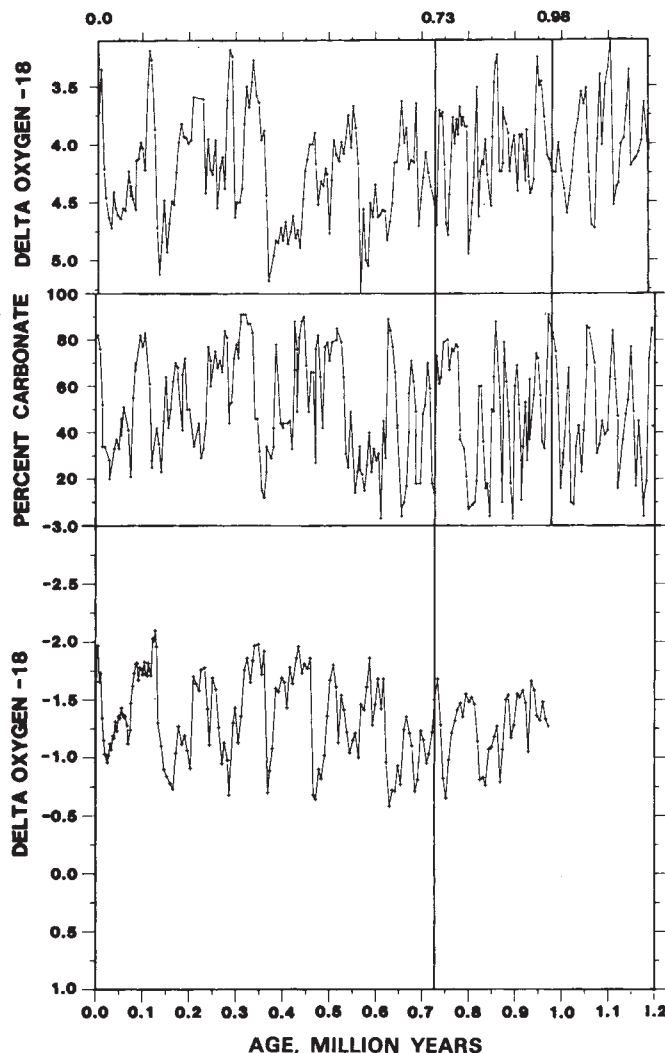


Fig. 3 Top: Oxygen isotope record of Site 552A cores 1–5. The plotting scale is linear between magnetic reversal horizons. Middle: carbonate content in the same interval; low carbonate content implies dilution by ice-rafted material. Bottom: oxygen isotope record of Pacific core V28-238 (ref. 4) for comparison. Vertical lines indicate the horizons used for time control: top (zero age), base Bruhnes normal chron (0.73 Myr), base Jaramillo subchron (0.98 Myr).

is shown as an approximate age scale calibrated on the basis of the single magnetic reversal in this core. Thus the comparison shows that there have been quite marked accumulation rate variations in DSDP 552A. This is hardly surprising in view of the lithological variations that are evident. Nonetheless the oxygen isotope record of Site 552A is complete to the extent of preserving every isotope stage.

In Figure 4, the oxygen isotope data for the lower part of the sequence are shown and compared with the oxygen isotope data for piston core V28-179. (Figure 4 shows additional measurements that we have made in core V28-179. Note that the position

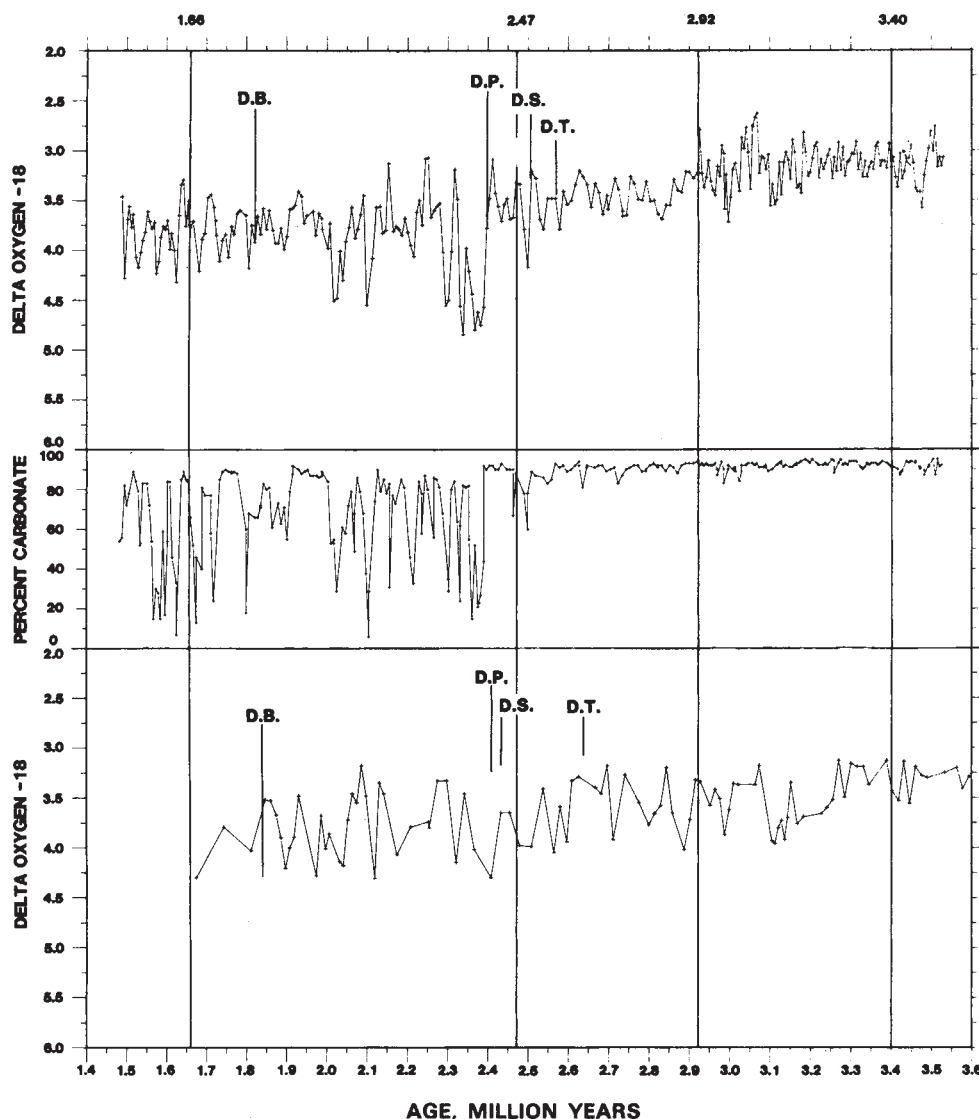


Fig. 4 Top: Oxygen isotope record of Site 552A cores 7–12. The plotting scale is linear between magnetic reversal horizons. Middle: carbonate content in same interval. Bottom: oxygen isotope record in Pacific core V28-179 (ref. 11) for comparison. Vertical lines show horizons used for time control: top Olduvai normal subchron (1.66 Myr), base Matuyama reversed chron (2.47 Myr), top Kaena reversed subchron (2.92 Myr), base Gauss normal chron (3.40 Myr). Nannofossil extinction horizons determined in both cores indicated by: DT, *Discoaster tamalis*; DS, *D. surculus*; DP, *D. pentaradiatus*; DB, *D. brouweri*.

of the Gauss–Matuyama boundary was incorrectly printed in ref. 11; it was in fact located at 1,338 cm, not 1,358 cm.) Again, to facilitate comparison, the plotting scale for both records has been adjusted at magnetostratigraphic boundaries as indicated, so as to approximate an age scale using a palaeomagnetic time scale¹². Obviously the application of this age scale becomes less accurate far from the magnetic reversals. However, comparison between the two records is good considering the low accumulation rate, and relatively coarse sampling interval in V28-179, and many small-scale features can be correlated between the two. The amplitude of variation is substantially greater in Site 552A than in V28-179. This is probably due to the effect of bioturbation in the Pacific core; since the accumulation rate of core V28-179 was only about 0.55 cm kyr^{-1} , one would expect climatic extremes lasting only a few thousand years to be severely degraded by bioturbation.

The series of nannofossil extinction data shown in Fig. 4 have also been determined quantitatively in core V28-179 (ref. 13) and their ages estimated. Using these estimates, the average accumulation rate over the pre-ice-raftering interval in Site 552A may be estimated at approximately 1.7 cm kyr^{-1} , surprisingly close to the average for the upper part, 1.8 cm kyr^{-1} (although the rate has certainly varied over short intervals during the intense climatic fluctuations of the past few million years). Palaeomagnetic stratigraphy and nannofossil stratigraphy in this site give identical estimates for the age of the first major northern hemisphere glacial event about 2.37 Myr.

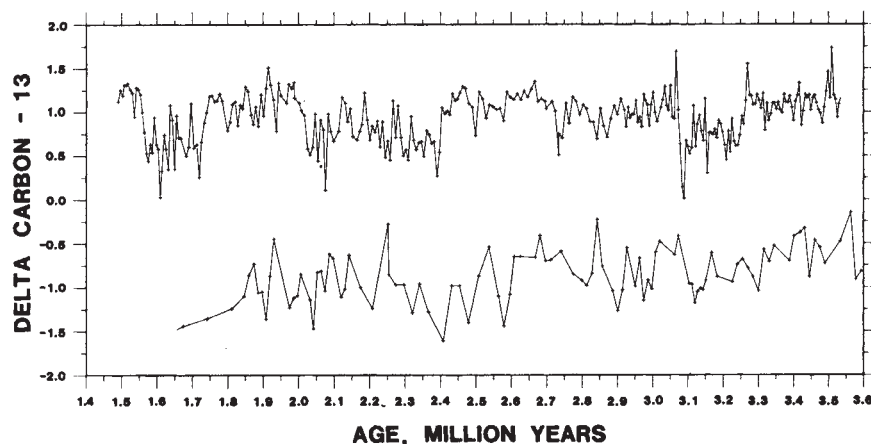
It is clear from an examination of Figs 3 and 4 that there is a remarkable correspondence between the records of oxygen isotopic composition in the foraminifera, and carbonate content, back to about 2.4 Myr. This is not particularly surprising since both are clearly causally related to glaciation. However, it is worth noting that if the late Pliocene isotopic fluctuations were attributed to glaciation in Antarctica, one would not expect the influx of ice-rafted debris during the glaciation at 2.4 Myr to be as dramatic as that observed in the last glacial, whereas this is what we observe in Site 552A. A brief ice-raftering episode, coinciding with positive ^{18}O values, is observed at about 2.5 Myr. Prior to 2.5 Myr, the influx of ice-rafted debris is minute and has little impact on the record of carbonate content. Presumably such glaciation as may have occurred before that time did not give rise to extensive calving of icebergs into the North Atlantic.

If we compare the isotopic composition of benthonic foraminifera at the level of the first glacial maximum at 2.37 Myr with values in glacial levels in the upper part of the sequence, it becomes apparent that the early event represents a truly glacial interval, with an ice volume similar to maxima during the middle Pleistocene. This must, for instance, be correlative with a fully glacial environment in Britain although obviously the actual ice-margin positions cannot be determined from these data.

Other data relating to a major climatic threshold being crossed at this time comes from the Netherlands and New Zealand. In the Netherlands, Zagwijn¹⁴ has shown that there was a complete

C-13 DATA DSDP552A and V28-179

Fig. 5 Carbon isotope records for Site 552A cores 7-12 (top) and Pacific core V28-179 (bottom). Both are plotted on the same ^{13}C scale and represent estimates of the ^{13}C contents of intermediate depth (2,311 m) water in the North Atlantic (above) and of deep (4,509 m) water in the equatorial Pacific (below). Today there is an observed difference of a little over 1% which reflects the fact that ocean deep waters are ventilated in the North Atlantic.



overturn in vegetational type just after the Gauss-Matuyama boundary; indeed he regards this as representing the Pliocene-Pleistocene boundary. In New Zealand, Stipp and others¹⁵ showed that there was a major drop in sea level between about 2.4 and 2.6 Myr; again, they correlated this event, which they interpreted as the first glacio-eustatic fall in sea level, with the Pliocene-Pleistocene boundary. We disagree with these opinions, only because we consider it well established that the Pliocene-Pleistocene boundary at the newly proposed stratotype at Vrica¹⁶, as well as at previous Calabrian sections, has a much younger age of about 1.6 Myr (ref. 17). If the boundary were selected on the basis of a significant climatic event rather than on historical precedent, it might appropriately be re-positioned so as to coincide with the climatic change about 2.4 Myr, but we do not recommend such an approach to the definition of geological boundaries.

The data shown in Fig. 4 also indicate that there was considerable climatic variability, somewhere on the globe, even before 2.5 Myr. It is well established that glaciation in Iceland occurred as early as 3.1 Myr (ref. 18) although recent work suggests that glaciation did not reach sea level in Iceland until about 2.0 Myr (ref. 19) and there is also evidence for glaciation in the Sierra Nevada, California, at about the same time²⁰. However, palaeoceanographic conditions on Rockall Bank do not suggest severe climates in North-west Europe. Occasional grains of ice-rafted debris do occur, and the nanofossil floras certainly show marked variation, but the scale of variation was much less before 2.4 Myr than it was after the event at that time.

Figure 5 shows the carbon isotope records for DSDP 552A and V28-179. The data are plotted on the same isotope scale and the same species-dependent correction factors have been made to both. The persistent isotopic difference between the two records of over 1% indicates a continuing situation in which the water bathing the sea floor on Rockall was isotopically heavier for ^{13}C than deep water in the Pacific, as is the case today. This difference reflects the difference between newly-formed, oxygen rich North Atlantic deep water (NADW) and older, oxygen-depleted deep Pacific water^{21,22}. We (and also Blanc *et al.*²³) disagree with Keigwin's deduction²⁴ that the formation of NADW only began with the closing of the Panama Straits at 3.0 Myr, although this event may well have affected the contribution that this water mass made to the deep water of the Caribbean deep waters that were the topic of Keigwin's study²⁴.

In the upper part of the section, it is clear that DSDP Site 552A preserves the whole of the standard oxygen isotope

record of the past million years. Although the fine structure of many stages is obscure, every single stage is present. By comparison, we note that the stratigraphically longest piston core from the North Atlantic, core Kane 708-7 (ref. 9), extends only to Stage 17 at about 0.6 Myr. For the earlier record, only DSDP Site 116, recently re-investigated from the palaeoclimatic point of view²⁵, was previously available. Site 116 was recovered by conventional rotary drilling and the sediment was so disturbed that glacial and non-glacial materials are intermixed, precluding any high-resolution studies being made. It is to be hoped that the new technique of ^{13}C analysis in the organic carbon portion of the sediment, pioneered in Site 116 (ref. 25), will be applied to Site 552A. We believe that the material recovered in Site 552A is ideal for detailed studies of climatic evolution in the ocean adjacent to the north-west European region where the Plio-Pleistocene continental record is best known.

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1. Berggren, A. A. *Init. Rep. DSDP* **12**, 953-963 (1972).
2. Backman, J. *Stockh. Contr. Geol.* **32**, 113-137 (1979).
3. Prell, W. L. *et al. Init. Rep. DSDP* **68**, 5-6 (1982).
4. Shackleton, N. J. & Opdyke, N. D. *Quat. Res.* **3**, 39-55 (1973).
5. Shackleton, N. J. *Coll. int. Cent. natn. Rech. Scient.* **219**, 203-210 (1974).
6. Graham, D. W., Corliss, B. H., Bender, M. L. & Keigwin, L. D. Jr *Mar. Micropalaeont.* **6**, 483-497 (1981).
7. Duplessy, J.-C. *et al. Quat. Res.* (in the press).
8. Goree, W. S. & Fuller, M. *Rev. Geophys. Space Phys.* **14**, 591-608 (1976).
9. Ruddiman, W. F. & McIntyre, A. *Mem. Geol. Soc. Am.* **145**, 111-146 (1976).
10. Imbrie, J. *et al. in Milankovich and Climate* (eds Berger, A. L. *et al.*) (Reidel, Dordrecht, in the press).
11. Shackleton, N. J. & Opdyke, N. D. *Nature* **270**, 216-219 (1977).
12. Berggren, W. A., Kent, D. V. & van Couvering, J. *Geol. Soc. Lond. Spec. Publ.* (ed. Snelling, N. L.) (in the press).
13. Backman, J. A. & Shackleton, N. J. *Mar. Micropalaeont.* **8**, 141-170 (1983).
14. Zagwijn, W. *Boreas* **3**, 75-97 (1974).
15. Stipp, J. J., Chappell, J. H. A. & McDougall, I. *Am. J. Sci.* **265**, 462-474 (1967).
16. Selli, R. *et al. Giorn. Geol.* **42**, 181-204 (1977).
17. Backman, J., Shackleton, N. J. & Tauxe, L. *Nature* **304**, 156-158 (1983).
18. McDougall, I. & Wensink, H. *Earth planet. Sci. Lett.* **1**, 232-236 (1966).
19. Albertsson, K. J. *Náttúrufræðingurinn* **48**, 1-8 (1978).
20. Curry, R. R. *Science* **154**, 1121-1142 (1966).
21. Duplessy, J.-C. thesis, Univ. Paris VI (1972).
22. Kroopnick, P., Deuser, W. G. & Craig, H. *J. geophys. Res.* **75**, 7668-7671 (1970).
23. Blanc, P. L., Rabussier, D., Vergnaud-Grazzini, C. & Duplessy, J.-C. *Nature* **283**, 553-555 (1980).
24. Keigwin, L. D. *Science* **217**, 350-353 (1982).
25. Blanc, P. L., Fontugne, M. R. & Duplessy, J.-C. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **42**, 211-224 (1983).

Seasonal fluctuations in deep-sea sediment community oxygen consumption: central and eastern North Pacific

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Historically the deep sea was viewed as a homogeneous environment in time and space characterized by high hydrostatic pressure, low temperature and low food supply^{1,2}. The highly diverse but low-density communities of organisms^{3,4} occupying this environment were dependent on a slow, constant rain of small particulate organic matter from the overlying surface water for food. This material slowly settled through thousands of metres of the water column, dampening any pulses in primary production at the surface. The rate at which this 'constant' food source was used was found to be slow⁵⁻⁸. However, there was early evidence⁹⁻¹², with much recent support¹³⁻¹⁷, that some deep-sea animals exhibited seasonal reproduction. In addition, the flux of small particulate organic matter to the deep-sea floor has been shown to be seasonal and in phase with rates of surface primary productivity¹⁸⁻²⁰. We report here the first evidence of seasonality in biological rates in the deep sea: *in situ* rates of sediment community oxygen consumption at two abyssal stations in the central and eastern North Pacific are highest in early summer, decreasing to the lowest rates in late autumn and winter.

We have measured sediment community oxygen consumption (SCOC) seasonally at two stations over the past 6 yr: Station CNP in the oligotrophic central North Pacific north of Hawaii (31° N, 159° W; water depth 5,900 m), and Station C in the eutrophic eastern North Pacific, west of the southern California Continental Borderlands (32°33' N, 120°31' W; water depth 3,815 m).

Sediment community oxygen consumption was measured *in situ* using a free vehicle grab respirometer (FVGR)^{21,22}. This instrument consists of an integrated flotation and instrument tripod which is capable of continuously measuring the dissolved oxygen consumed by the undisturbed sediment and overlying water in four replicate grabs (413 cm² each). Polarographic oxygen sensors measure the dissolved oxygen tension in each grab with amplified outputs continuously recorded in the instrument package. Each sensor is calibrated using chilled saturated and nitrogen-purged seawater as limits and corrected for hydrostatic pressure effects and electrode oxygen consumption. Initial and final control bottom-water samples were incubated on the FVGR for each deployment (≤ 2.7 days at Station C, ≤ 4.9 days at Station CNP). On recovery of the FVGR, the undisturbed sediment with overlying water was analysed for macrofauna abundance and biomass, and organic carbon and total nitrogen. Total oxygen consumption rates, corrected for the overlying water rates in each grab, yielded SCOC rates²³. Sediments in two or three grabs from each FVGR deployment were sieved through a 297- μ m mesh screen on board ship and the abundance and wet weight biomass of the retained macrofauna determined in the laboratory. Small aliquots of surface sediment (0-1 cm) from each grab were frozen for CHN analysis in the laboratory where these samples were acidified with 0.1 M HCl, dried, and three replicate samples analysed using a Perkin-Elmer 240C CNH analyser.

Characterization of each bottom station is presented in Table 1. Variations of the sediment biological and chemical parameters measured are large and may represent intra-station heterogeneity.

Rates of SCOC at both stations were significantly higher ($P < 0.05$) in June than in November (Station CNP) and

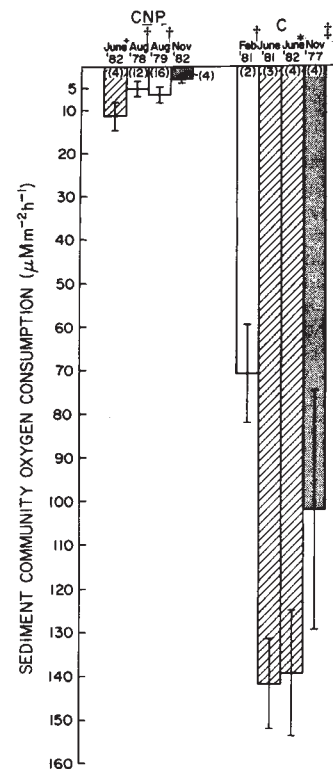


Fig. 1 Sediment community oxygen consumption measured *in situ* on a seasonal basis at Stations CNP and C. Mean rates with standard deviations are presented; the number of replicates is given in parentheses. * † ‡ Values reported previously (refs 23, 24 and 21, respectively).

February (Station C) (Fig. 1). At Station CNP, June rates of SCOC were almost four times higher than in November. The high and low measured rates of SCOC in June and November bracketed intermediate values measured in August over two consecutive years. The non-significant difference ($P > 0.05$) between the SCOC rates measured in August of 1978 and 1979 suggests a repeatable pattern.

Although substantially higher rates of SCOC were measured at Station C, a pattern similar to Station CNP is exhibited. Mean rates of SCOC are twice as high in June as in February (Fig. 1) with intermediate rates measured in November. These seasonal measurements of SCOC at both stations fall within the range of values measured sporadically at various depths and temperatures in the world oceans²⁴⁻²⁸.

We propose that the fluctuations in SCOC measured at three different times of the year at both stations reflect seasonal variations in biological rates in the deep sea. The logical question is what causes such fluctuations in a seemingly constant environment. On the hypothesis²⁹ that the environment is food-limited, the most obvious answer is food supply; food energy used by these soft-bottom abyssal communities is ultimately derived from primary production in the euphotic zone. On a variety of temporal scales, a fraction of this production must reach bottom waters directly by sinking, diffusion and advection, indirectly by trophic transfers, or by a combination of both. This food can exist in three forms: (1) small particulate organic matter, (2) large organic falls and (3) dissolved organic matter.

(1) The flux of small particulate organic matter has not been measured seasonally in our study areas. However, a correlation has been shown between the annual cycle of surface primary productivity and the flux of small particulate organic matter reaching abyssal depths in the western North Atlantic¹⁸ and eastern tropical Pacific¹⁹. The close synchrony between the annual cycles of surface primary productivity and particulate organic matter collected by sediment traps at 3,200 m (ref. 18) and 3,560 m (ref. 19) suggests rapid settling rates (> 65 m per

Table 1 Characterization of two stations in the North Pacific, C and CNP

Station parameters	Station CNP				Station C			
	June 1982	August 1978*	August 1979*	November 1982	February 1981*	June 1981	June 1982	November 1977†
Bottom water								
Dissolved O ₂ (μmol)	167‡	167*	168*	171	138*	162	138‡	172‡
Temperature (°C)	1.6‡	1.5*	1.5*	1.6	1.6*	1.6	1.6‡	2§
Sediments								
Sediment type	Clay‡	Clay*	Clay*	Clay	Silty clay*	Silty clay	Silty clay‡	Silty clay†
Surface organic carbon (mg C g ⁻¹)	3.50‡	4.20 ± 2.08 (3)*	5.40 ± 3.24 (3)*	3.40	13.7*	11.70	15.70‡	12.24 ± 0.11 (2)†
Surface total nitrogen (mg N g ⁻¹)	0.20‡	0.50 ± 0.44 (3)*	0.47 ± 0.25 (3)*	1.10	1.4*	1.30	1.00‡	1.74 ± 0.14 (2)†
Macrofaunal abundance (individuals m ⁻²)	752 ± 103 (2)	201 ± 189 (6)*	584 ± 272 (8)*	497 ± 223 (2)	8,697 ± 1,179 (3)*	7,202 ± 868 (3)	4,695 ± 1,115 (2)	5,274 ± 3,115 (2)†
Macrofaunal biomass (mg wet wt m ⁻²)	30.94 ± 6.71 (2)	61.5 ± 74.9 (6)*	20.6 ± 39.1 (8)*	50.7 ± 61.8 (2)	664.8 ± 423.0 (3)*	1,713.4 ± 1,060.6 (3)	1,936.0 ± 526.5 (2)	1,539.0 ± 531.1 (2)†

Means ± s.d. are presented where more than one grab was sampled (number of replicates in parentheses). Macrofaunal biomass for Station C in November excludes two large animals per grab—inclusion of these animals would increase biomass to 24,190 ± 13,407.0 mg wet wt m⁻².

* ‡ Values previously reported (refs 24, 21 and 23, respectively).

§ Value rounded to nearest degree (ref. 21).

day) in both areas^{19,30}. More precise estimates of sedimentation rates may be determined using time-lapse photography. Billet *et al.* report rates of 100–150 m per day in the north-east Atlantic²⁰. Although complete seasonal measurements of primary productivity are lacking for our two stations, data are available for areas in close proximity.

Seasonal rates of surface primary productivity are well documented for the Southern California Bight, an area 17 miles inshore of Station C. Our high rates of SCOC in June and low rates in February correlate well with the seasonal productivity maxima between March and July³¹, allowing for sinking rates of ≤38 days²⁰ over the 3,800-m water column.

A less clear picture of primary productivity exists for the central North Pacific. Early estimates of productivity in the central gyre area recorded winter peaks in primary productivity corresponding to increased mixing of subsurface nutrient-rich water with surface water³². Although Hayward and associates³³, on analysing recent ¹⁴C measurements at a station south of Station CNP, found no obvious trends in primary productivity, seasonal variation may be masked by the large intraseasonal variability. One indication of seasonal variation is the existence of a high subsurface oxygen maximum in this same area in summer, which, if photosynthetically produced, would yield substantially higher estimates of primary productivity than indicated by the ¹⁴C method³⁴. Of the three times of the year measured, SCOC at Station CNP was highest in June and lowest in November (Fig. 1). If we assume that the photosynthetically produced particulate organic matter which escapes the euphotic zone sinks at a rate of 100–150 m per day (ref. 20), then one would expect this food supply to reach the bottom community within 2 months. Therefore, our data predict that peaks in primary productivity would occur in spring. However, faster sinking rates up to 1,028 m per day have been reported for zooplankton and midwater fish faecal pellets^{35,36} yielding bottom arrival times of less than 10 days at 5,900 m.

(2) The contribution of large organic falls (for example, carcasses of pelagic fishes and squid, shallow benthic and terrestrial plant remains) to the food supply of the deep sea is unknown³⁷. Large pelagic fishes such as tuna, *Thunnus alalunga*, undergo seasonal migrations in the North Pacific with high numbers occurring in winter in the vicinity of Station CNP, and in the spring and early summer in the vicinity of Station C^{38,39}. Although seasonal mortality rates are unknown for these and other large pelagic animals, the potential importance of this food source would increase during periods of maximum abundance. In addition, Station C may be influenced by seasonal inputs of drift kelp; peak kelp abundances appear on beaches and probably offshore during the stormy winter months⁴⁰.

(3) Although dissolved organic matter (DOM) represents a large pool of potential food in the deep sea, measurements of DOM in the central and eastern North Pacific near the sediment–

water interface reveal this source to be 'old' and refractory^{41,42}. However, seasonal measurements of combined dissolved and particulate organic carbon at 300–1,500 m depth in the central Pacific do show significantly higher concentrations in the winter than in summer⁴³.

The seasonality of any of these potential food sources may be influenced more directly by abyssal advection than by surface and atmospheric conditions. Abyssal currents have been shown to have seasonal signals in the eastern North Atlantic, generated by high surface wind stress in winter with temporal lag and decay in magnitude with depth⁴⁴. This, coupled with observations of temporal variability in the nepheloid layer at abyssal depths in the western North Atlantic⁴⁵, suggests the potential impact of seasonal pulses on abyssal advection of food.

Our results suggest a seasonal pattern in SCOC which is related to the seasonal productivity regime of the overlying waters in the eutrophic eastern boundary current region (Station C) and possibly in the oligotrophic central gyre region (Station CNP). Deep-sea areas can no longer be considered to be in static equilibrium exhibiting constant biological rates, but must be examined as components in dynamic equilibrium with the overall open ocean ecosystem. Mass balance calculations of organic matter remineralization which are based on a single measurement in time must be re-evaluated considering fluctuations which in the case of SCOC can vary by as much as a factor of four between seasons.

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1. Bruun, A. F. *Mem. geol. Soc. Am.* **67**, 641–672 (1957).
2. Menzies, R. J. *Oceanogr. mar. Biol. A. Rev.* **3**, 195–210 (1965).
3. Sanders, H. L., Hessler, R. R. & Hampson, G. R. *Deep Sea Res.* **12**, 845–867 (1965).
4. Hessler, R. R. & Sanders, H. L. *Deep Sea Res.* **14**, 65–78 (1967).
5. Jannasch, H. W., Eimhjellen, K., Wirsén, C. O. & Farmanfardian, A. *Science* **171**, 672–675 (1971).
6. Jannasch, H. W. & Wirsén, C. O. *Science* **180**, 641–643 (1973).
7. Smith, K. L. Jr & Teal, J. M. *Science* **179**, 853–858 (1973).
8. Turekian, K. K. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 2829–2833 (1975).
9. Mead, G. W., Bertelsen, E. & Cohen, D. *Deep Sea Res.* **11**, 569–596 (1964).
10. George, R. Y. & Menzies, R. J. *Nature* **215**, 878 (1967).
11. George, R. Y. & Menzies, R. J. *Nature* **220**, 80–81 (1968).
12. Schoener, A. *Ecology* **49**, 81–87 (1968).
13. Rokop, F. J. *Science* **186**, 743–745 (1974).
14. Rokop, F. J. *Mar. Biol.* **43**, 237–246 (1977).
15. Lightfoot, R. H., Tyler, P. A. & Gage, J. D. *Deep Sea Res.* **26A**, 967–973 (1979).
16. Tyler, P. A., Grant, A., Pain, S. L. & Gage, J. D. *Nature* **300**, 747–750 (1982).
17. Gage, J. D. *Deep Sea Res.* **29**, 1565–1586 (1982).
18. Deuser, W. G. & Ross, E. H. *Nature* **283**, 364–365 (1980).
19. Honjo, S. *Science* **218**, 883–884 (1982).
20. Billett, D. S. M., Lampitt, R. S., Rice, A. L. & Mantoura, R. F. C. *Nature* **302**, 520–522 (1983).
21. Smith, K. L. Jr, White, G. A. & Laver, M. B. *Deep Sea Res.* **26A**, 337–346 (1979).
22. Smith, K. L. Jr & Baldwin, R. J. in *Handbook on Polarographic Oxygen Sensors* (eds Gnaiger, E. & Forstner, H.) 298–319 (Springer, Berlin, 1983).
23. Smith, K. L. Jr & Laver, M. B. *Limnol. Oceanogr.* (submitted).
24. Smith, K. L. Jr, Laver, M. B. & Brown, N. O. *Limnol. Oceanogr.* **28**, 882–898 (1983).

25. Smith, K. L. Jr *Limnol. Oceanogr.* **19**, 939–944 (1974).
26. Smith, K. L. Jr *Mar. Biol.* **47**, 337–347 (1978).
27. Hinga, K. R., Sieburth, J. McN. & Heath, G. R. *J. mar. Res.* **37**, 557–579 (1979).
28. Murray, J. M. & Grundmanis, V. *Science* **209**, 1527–1530 (1980).
29. Dayton, P. K. & Hessler, R. R. *Deep Sea Res.* **19**, 199–208 (1972).
30. Deuser, W. G., Ross, E. H. & Anderson, R. F. *Deep Sea Res.* **28A**, 495–505 (1981).
31. Smith, P. E. & Eppley, R. W. *Limnol. Oceanogr.* **27**, 1–17 (1982).
32. McGowan, J. A. in *Biology of the Oceanic Pacific* (ed. Miller, C. B.) 9–28 (Oregon State University, Corvallis, 1974).
33. Hayward, T. L., Venrick, E. L. & McGowan, J. A. *J. mar. Res.* **41**, 711–729 (1983).
34. Schulenberger, E. & Reid, J. L. *Deep Sea Res.* **28A**, 901–919 (1981).
35. Komar, P. D., Morse, A. P., Small, L. F. & Fowler, S. W. *Limnol. Oceanogr.* **26**, 172–180 (1981).
36. Robison, B. H. & Bailey, T. G. *Mar. Biol.* **65**, 135–142 (1981).
37. Stockton, W. L. & DeLaca, T. E. *Deep Sea Res.* **29A**, 157–169 (1982).
38. McGary, J. W., Graham, J. J. & Otsu, T. *Calif. Coop. Oceanic Fish. Invest. Rep.* **8**, 45–53 (1961).
39. Laurs, R. M. & Lynn, R. J. *Fish. Bull.* **75**, 795–822 (1977).
40. Zobell, C. E. *Nova Hedwigia* **32**, 269–318 (1971).
41. Williams, P. M., Carlucci, A. F. & Olson, R. *Oceanol. Acta* **3**, 471–476 (1980).
42. Williams, P. M., Stenhouse, M. C., Druffel, E. M. & Koide, M. *Nature* **276**, 698–701 (1978).
43. McGowan, J. A. & Williams, P. M. *J. exp. Mar. Biol. Ecol.* **12**, 187–217 (1973).
44. Dickson, R. R., Gould, W. J., Gurbutt, P. D. & Killworth, P. D. *Nature* **295**, 193–198 (1982).
45. Gardner, W. D. & Sullivan, L. G. *Science* **213**, 329–331 (1981).

Phosphorus limitation of net production in a confined aquatic ecosystem

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The sediment composition of confined aquatic ecosystems with little terrigenous input reflects net ecosystem production. Here we present nutrient budgets for one such system, Shark Bay in Western Australia, which suggest that net ecosystem production is limited by the oceanographic delivery of P, while the system meets its N requirements by nitrogen fixation. We suggest that N versus P limitation of the net production of aquatic ecosystems reflects the degree of confinement of those systems.

An aquatic ecosystem isolated from exogenous inputs of organic material must have a long-term net organic production rate which equals or exceeds zero. Were such an isolated system not biased towards net autotrophy, the biomass and sedimentary organic materials would be consumed and the system would eventually collapse. If the system also does not export organic material, then the sediments must reflect the long-term net production of organic material. While these relationships must be true in principle, they rarely have application: organic additions or losses usually obscure the sedimentary record of net organic metabolism.

Shark Bay, Western Australia (Fig. 1), is a large (13,000 km²), seagrass-dominated coastal lagoon¹ which might be expected to preserve this theoretical relationship between net organic production and sediment composition. The bay is surrounded by desert; there is virtually no input of fresh water via runoff, rainfall or groundwater. Exchange of materials with the ocean is slow; average water residence time in the bay, estimated from water and salt budgets¹ exceeds 1 yr.

Net uptake of dissolved inorganic materials from incoming water, although very slow, nevertheless drastically modifies the water composition. This modification represents the net signal of biogeochemical fluxes, integrated over the long water residence time¹. For example, ocean water entering the bay has a reactive P concentration of ~0.2 mmol m⁻³; reactive P in the bay decreases to ~0.03 mmol m⁻³.

We refer to budgets of net C and P uptake derived from a box model of water and salt balance as the 'oceanographic

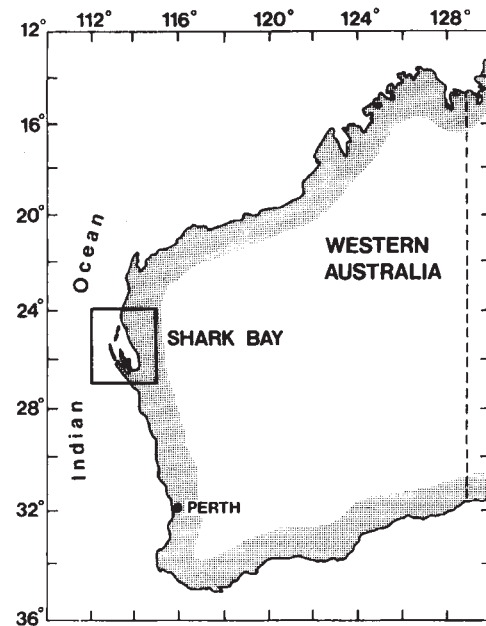


Fig. 1 Location map of Shark Bay, Western Australia.

budgets'. The rate term for these budgets is derived from net evaporation rates¹. Because of the virtual isolation of the bay from particulate inputs and outputs, we hypothesized that the sediments should reflect the net production. We refer to the sedimentary record of net production as the 'sediment budgets'. Because the absolute sedimentation rate of Shark Bay is unknown, we do not have a normalizing parameter for the sediment budgets. We therefore compare data for the oceanographic and sediment budgets by means of material ratios, thus removing the absolute rate terms from both sets of budgets.

Water loss from net evaporation is compensated by net advective (tidal) inflow. The salt balance is maintained by advective inflow and loss by horizontal eddy diffusion. Deviation of the concentrations of other chemical constituents from proportionality with salinity is attributed to net uptake.

Table 1 is a summary of net uptake of C into CaCO₃ and the net uptake of both C and reactive P into organic materials, as derived from the oceanographic budgets. Note that 27% of the carbon taken up enters the organic fraction and the remainder enters CaCO₃. Moreover, the molar ratio of C to P taken up by the net production of organic materials is ~320:1.

Sediment grab samples were collected along the same transect through the eastern gulf of Shark Bay as was used for the oceanographic budgets. Samples were dried at 110 °C. Weighed aliquots were muffled at 550 °C for 2 h, re-weighed, remuffled at 1,000 °C, and weighed again. Combustion at 550 °C oxidizes organic materials². Organic carbon was calculated as 40% of the weight loss at 550 °C. Combustion of CaCO₃ at 1,000 °C drives off CO₂. Inorganic carbon was calculated to be 27% of this latter weight loss.

This procedure gives results more reproducible for the C composition of organic-poor calcareous sediments than do CHN analyses according to the technique of Hirota and Szyper³. We found no evidence of significant carbonate loss at 550 °C, although some Mg calcite loss could have escaped our notice².

Total sediment N was determined by standard colorimetric techniques after sulphuric acid Kjeldahl digestion of samples, and total P was determined colorimetrically after sample digestion with nitric and perchloric acids.

Table 2 summarizes sediment composition. The inorganic C content of the sediments averages 8.2 mmol g⁻¹ (82% CaCO₃). The average organic C content of the sediments is 2.1 mmol g⁻¹

Table 1 Net rates of carbon and phosphorus uptake by biogeochemical processes in the eastern gulf of Shark Bay

Process	Rate (mmol per m ² per day)
CaCO ₃ production	3.2
Organic carbon production	1.2
Phosphorus uptake	0.0037

Rates derived from Smith and Atkinson¹.

(6% organic material). Thus, no more than 12% of the sediments come from non-carbonate, inorganic sources.

Organic C represents 20% of the C in the sediments, about one-quarter lower than the organic fraction of C uptake derived from the oceanographic budgets (Table 1). Broecker and Takahashi⁴ were unable to reconcile an oceanographically determined C budget for the Bahama Banks with the organic C content of sediments there. In most settings where the biomass in the system studied exceeds that of the surrounding waters, there may be significant net export of organic materials by advective and mixing losses and by the migration of organisms. Extremely slow water exchange and apparently small migration fluxes obviate such a problem in Shark Bay. Even so, the slight discrepancy between the organic C fraction of the oceanographically determined C uptake and the organic C fraction of the sediments may represent loss of organic materials.

The average P content of the sediments is 0.007 mmol g⁻¹, and the sediment molar ratio of organic C to P is 300:1 (Table 2), not significantly different from the uptake ratio derived from the oceanographic budget (Table 1). From the correspondence between the oceanographic and sediment budgets for C and P, we are confident that the sediments adequately reflect net ecosystem uptake. Sediment N composition should therefore reflect net N uptake. The sediment N content is 0.05 mmol g⁻¹ (Table 2), and the molar C:N:P ratio of the sediments is ~300:7:1.

We now use the oceanographically derived rates of C and P uptake and the sediment C:N:P ratio to scale N uptake, and we derive a rate of 0.03 mmol per m² per day. If an error exists in this estimate, it should be of the same order as the discrepancy between the oceanographic and sediment C budgets (<25%). The net N uptake by this system is <0.04 mmol per m² per day.

Nitrate plus ammonia concentration throughout the bay is less than 0.5 mmol m⁻³, and there is no coherent pattern of spatial variation (Smith and Atkinson, unpublished data). Applying this average concentration and a nitrogen/salinity gradient of 0 to our box model¹ yields an oceanographic supply of N of 0.002 mmol per m² per day; less than 10% of the N being taken up into the sediments can be supplied by oceanographic input. The remainder must enter by other pathways, with atmospheric N fixation being the most likely one.

We did not measure N fixation, nor do we believe that such measurements are directly relevant. It would be impossible to estimate the N fixation rate for such a large, heterogeneous area from a few spot measurements of local N fixation, and the phenomenon of N fixation is adequately documented. Capone and Carpenter⁵ report N fixation rates of 1–0.02 mmol per m² per day for environments ranging from seagrass beds to bare estuaries and shallow benthic environments. Our initial estimate of N fixation in Shark Bay falls near the low end of this range.

The sedimentary end product of Shark Bay organic materials appears greatly depleted with respect to N compared with the N/P ratios of materials being produced. The low sediment N/P ratio (7:1) compared with either plankton (16:1 (ref. 6); confirmed with our own data for Shark Bay) or benthic plants (30:1 (ref. 7); confirmed for Shark Bay) suggests that N is more readily

Table 2 Sediment composition in the eastern gulf of Shark Bay

Component	Amount (mmol g ⁻¹)
Inorganic C	8.2 ± 0.18 (38)
Organic C	2.1 ± 0.10 (39)
Total N	0.05 ± 0.06 (39)
Total P	0.007 ± 0.005 (39)

Values are the mean ± s.d. (n).

released from particulate organic materials in the sediments than are P and C. This release may represent denitrification⁸. In that event, our estimate of N fixation might be too low by 2–4-fold. The upper limit of possible N fixation in Shark Bay (<0.16 mmol per m² per day) is still well within the range for comparable environments elsewhere⁵.

We offer the following simple model to explain our observations. The two primary components to the model are the flushing rate of water through the system and the presence of N fixation as a supplement to the oceanographic (and, in other systems, land-derived) N sources.

As summarized above, marine plants ordinarily contain 16 to 30 times as much N as P, yet surface seawater typically has an inorganic N/P ratio of ≤10. Surface seawater is thus deficient in N, relative to the nutritional requirements of plants⁹. A highly productive environment subjected to such N-deficient water may fix N (for example, coral reef flats¹⁰); however such open environments export the locally fixed N (ref. 11) before the supply of P has been lowered measurably¹². By contrast, confined systems such as Shark Bay will accumulate fixed N. They supplement their N deficiency until P is exhausted. The system-wide rates required to exhaust available P are small relative to documented community or organismic N fixation rates⁵. Therefore the systems clearly have the capacity to 'fine-tune' N fixation rates to use available P. The supply of N is effectively limitless; the supply of P is not.

The apparent conflict between N and P limitation of net community production in aquatic systems can be resolved into extremes of a continuum. Many open systems receiving water which is deficient in N fail to accumulate N and are examples of open, N-limited systems⁹. The net community production of progressively confined systems will tend towards P limitation, because of accumulation and recycling of internally fixed N until P is exhausted. Isolated hypersaline coastal lagoons such as Shark Bay, some relatively enclosed coral atoll lagoons¹³, many lakes with long water-residence times¹⁴, and the whole of the world's oceans¹⁵ are examples of confined, P-limited ecosystems.

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1. Smith, S. V. & Atkinson, M. J. *Limnol. Oceanogr.* **28**, 625–639 (1983).
2. Krom, M. D. & Berner, R. A. *J. sedim. Petrol.* **53**, 660–663 (1983).
3. Hirota, J. & Szyper, J. P. *Limnol. Oceanogr.* **20**, 896–900 (1975).
4. Broecker, W. S. & Takahashi, T. *J. geophys. Res.* **71**, 1575–1602 (1966).
5. Capone, D. G. & Carpenter, E. J. *Science* **217**, 1140–1142 (1982).
6. Redfield, A. C., Ketchum, B. H. & Richards, F. A. in *The Sea* Vol. 2 (ed. Hill, M. N.) (Wiley, New York, 1963).
7. Atkinson, M. J. & Smith, S. V. *Limnol. Oceanogr.* **28**, 568–574 (1983).
8. Nixon, S. W. in *Estuaries and Nutrients* (eds Neilson, B. J. & Cronin, L. E.) 111–138 (Humana, Clifton, New Jersey, 1981).
9. Ryther, J. H. & Dunstan, W. M. *Science* **171**, 1008–1013 (1971).
10. Wiebe, W. J., Johannes, R. E. & Webb, K. L. *Science* **188**, 257–259 (1975).
11. Webb, K. L. *et al. Limnol. Oceanogr.* **20**, 198–210 (1975).
12. Pilson, M. E. O. & Betzer, S. B. *Ecology* **54**, 581–588 (1973).
13. Smith, S. V. *et al. Univ. Hawaii/Univ. South Pacific Int. Seagrass Prog. Tech. Rep.* (in the press).
14. Schindler, D. W. *Science* **195**, 260–262 (1977).
15. Broecker, W. S. & Peng, T.-H. *Tracers in the Sea* 690 (Eldigio, Palisades, New York, 1982).

Mallomonadacean microfossils provide evidence of recent lake acidification

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The Mallomonadaceae (Chrysophyceae), a group of flagellated algae commonly found in freshwater systems, are characterized by an armour of overlapping siliceous scales that are taxonomically diagnostic¹. Mallomonadacean scales are usually abundant and well preserved in lake sediments². Previous palaeolimnological studies have used the stratigraphic distribution of mallomonadacean assemblages to document patterns of lake eutrophication²⁻⁵. Our data demonstrate that the distribution of certain mallomonadacean taxa is also closely related to lakewater pH, that stratigraphic analyses of fossil scales can be used to indicate past changes in lake acidity, and that such stratigraphic studies provide important insights concerning the responses of sensitive lake systems to acid deposition.

Subfossil mallomonadacean assemblages from the surface sediments of 38 lakes in the Adirondack Mountains (New York, USA) have been studied with respect to the limnological factors that appear to influence the distribution of individual species and the characteristics of assemblages⁶. The Adirondack area is a largely forested and mountainous region that contains about 3,000 lakes and ponds, and is one of the most important lake regions in North America. The lakes are generally dilute and unproductive. The area receives intense acidic precipitation and, since many lakes have been affected^{7,8}, it is important that we be able to determine the recent acidification histories of sensitive lake systems. Detailed information on lake locations, morphometry, and chemistry has been presented elsewhere⁹.

Surface sediment samples (0–1 cm) were taken from the deepest areas of the study lakes using a modified Cushing and Wright piston corer¹⁰. Because mallomonadacean scales are siliceous, sediment digestions and microscopic techniques were the same as those used for fossil diatoms². Although electron microscopy is required to confirm taxonomic identifications, scales can usually be distinguished to the species level with light microscopy. An average of 600 scales was counted from each sediment sample. A total of 30 species from five genera was identified⁶.

Reciprocal averaging and cluster analyses indicated that pH and factors correlated with it exerted the greatest influence on

Table 1 Morphometric, physical and chemical characteristics of Deep Lake

Lake characteristic	
Surface area	11 hectare
Maximum depth	24 m
Elevation	789 m
Watershed area	72 hectare
pH	4.7
Alkalinity	−18 $\mu\text{equiv. l}^{-1}$
Conductivity	29 $\mu\text{S cm}^{-1}$ at 25 °C
Colour, true	7.5 Pt. Co units
Colour, Forel-Ule, median	7 units
Secchi disk transparency	8.4 m
P, total	3.1 $\mu\text{g l}^{-1}$
Chlorophyll <i>a</i>	1.7 $\mu\text{g l}^{-1}$
Ca	64 $\mu\text{equiv. l}^{-1}$
Mg	26 $\mu\text{equiv. l}^{-1}$
Na	13 $\mu\text{equiv. l}^{-1}$
K	6 $\mu\text{equiv. l}^{-1}$
SO ₄	150 $\mu\text{equiv. l}^{-1}$
Cl	7 $\mu\text{equiv. l}^{-1}$
NO ₃	12 $\mu\text{equiv. l}^{-1}$
Al, total	611 $\mu\text{g l}^{-1}$
SiO ₂	2.7 mg l^{-1}

Alkalinity, pH, conductivity, and true colour data are for surface (0.5 m) samples. Total P, chlorophyll *a*, cations, anions, Al, and SiO₂ data are for composite lake-average samples. All data are 1978–1979 summer (May–August) averages except for cation, anion, Al, and SiO₂ which are for samples taken 1 September, 1979.

mallomonadacean assemblages⁶. The distributions of individual taxa were plotted against each of the 21 limnological factors studied (as in Table 1). Scales of *Mallomonas hindonii* Nicholls and/or *M. hamata* Asmund (Fig. 1) were especially common in acidic lakes (Fig. 2). Notable exceptions were the three bog lakes (pH < 5.4) in which only trace populations were noted, and in the two highest elevation lakes (pH < 4.8) where scales of *M. hamata* were never observed. The distribution of these two taxa did not correlate closely with any of the other limnological factors studied. These data suggest that *M. hindonii* and *M. hamata* are reliable indicators of acidic non-bog conditions. In the few acidic lakes where neither of these two species were common, scales of *Synura echinulata* Korsh. were usually abundant.

Because many lakes in the Adirondacks have been affected by anthropogenic acidic precipitation⁷, a stratigraphic analysis of fossil Mallomonadaceae was initiated to establish whether these microfossils could be used to trace past changes in lakewater pH. Deep Lake (43°36'58" N, 74°39'52" W; Table 1) was chosen for this study because of its suitability for such a palaeological study. It is a headwater lake with a deep cup-shaped basin, it is at a relatively high elevation (789 m), is acidic (pH = 4.7), and is similar to other lakes in the area that have recently become more acidic⁸. The drainage basin is underlain by granitic gneiss bedrock that is covered by thin tills and soils. Watershed vegetation is a mixed deciduous-coniferous forest consisting largely of sugar maple (*Acer saccharum* Marsh.), yellow birch (*Betula alleghaniensis* Britton.) and American beech (*Fagus grandifolia* Ehr.), with lesser amounts of red maple (*Acer rubrum* L.) and red spruce (*Picea rubens* Sarg.). The area was logged in the late 1800s, and then again in the early 1950s (F. Lamphear, personal communication). Our unpublished palynological data do not indicate any marked differences in vegetation succession between these two episodes of disturbance. The watershed is in the West Canada Creek Wilderness Area and contains no cultural development other than hiking trails. Historical data indicate that Deep Lake probably became more acidic sometime between the 1930s and the mid-1950s. Professional guides reported good fishing for brook trout (*Salvelinus fontinalis*) in Deep Lake during the 1930s. However, although Deep Lake was stocked annually with brook trout fingerlings between 1951 and 1968, only three trout were

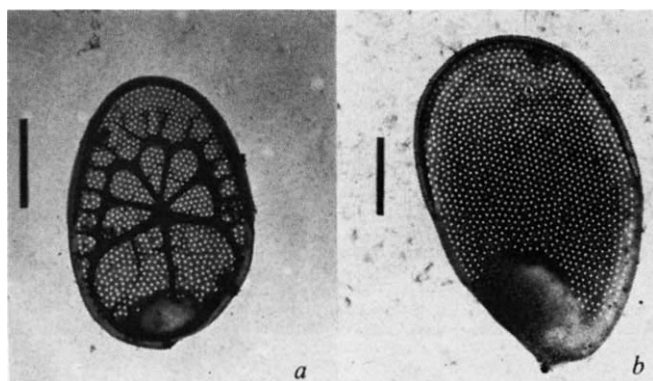
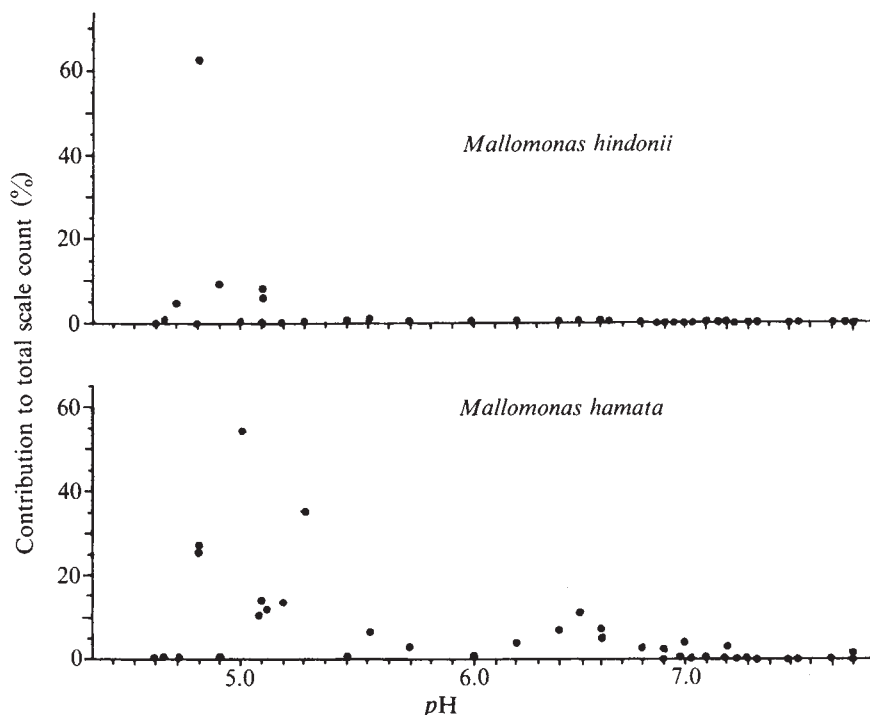


Fig. 1 Transmission electron micrographs of scales of *Mallomonas hindonii* (a) and *Mallomonas hamata* (b). Scale bar, 1 μm .

Fig. 2 The percentage contribution of *Mallomonas hindonii* and *M. hamata* scales plotted relative to the pH of each of the 38 study lakes.



captured during fish surveys in 1954, 1964 and 1965. No fish were caught during 1966, 1967 and 1968 surveys¹¹. Because brook trout usually cannot survive in lakes with a pH < 5.0 and high Al concentrations¹², conditions that currently exist in Deep Lake (Table 1), lake acidification was probably responsible for the disappearance of fish populations.

A 60-cm-long core was removed from the centre of Deep Lake and sectioned in the field at 1 cm intervals. The rise in non-arboreal pollen [ragweed (*Ambrosia*) and other weedy taxa] was recorded at the 5–6 cm level (Fig. 3). This horizon can be dated circa AD 1850, since an examination of historical records indicates that land clearance in the region southwest of the Adirondacks intensified at this time. Although the scales of 13 mallomonadacean taxa were identified in the Deep Lake stratigraphy, only six species ever represented more than 2% of the total scale sum (Fig. 3). Throughout the lower part of

the core, assemblages are dominated by *Mallomonas crassiquama* (Asmund) Fott with lesser amounts of *M. punctifera* Korsh. and *M. acaroides* Perty em. Iwanoff. However, a significant change occurs near the 3 cm level. There is a marked increase in *M. hindonii* and *M. hamata*, and, to a lesser extent, *Synura echinulata*. No changes in Mallomonadaceae coincided with the rise in non-arboreal pollen.

We believe that this shift in species composition was in response to lake acidification. As previously noted, *M. hindonii* and *M. hamata* are indicative of acidic lakes. Among the 38 Adirondack study lakes, *Synura echinulata* scales were also most abundant in the surface sediments of acidic lakes (for example, 81% of the total scale sum in Lake Tear of the Clouds, pH = 4.6; 74% in Mountain Pond, pH = 4.7; 59% in Lake Colden, pH = 5.0). In contrast, *M. crassiquama*, which is the most widespread *Mallomonas* sp. in the world^{13–15}, occurred only rarely in

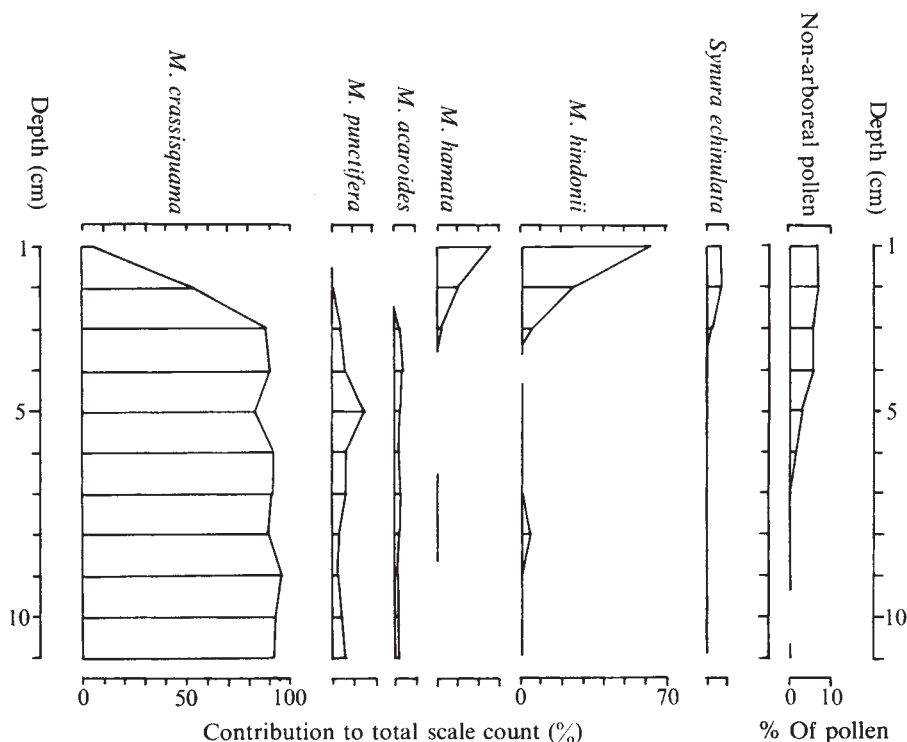


Fig. 3 Percentage diagram of the stratigraphic distribution of Mallomonadaceae in the Deep Lake sediments. The 15-cm and 20-cm levels were also analysed, but they were similar to the 11-cm level and are not presented here. An average of 600 scales was identified and enumerated from each level. *Mallomonas punctifera* Korsh. is synonymous with *M. pulchella* (Kisselev) Cronberg & Kristiansen and *M. reginae* Teil¹⁵.

assemblages from lakes with a $pH < 5.0$. A similar distribution for this taxon has been reported in other areas¹³. Similarly, *M. punctifera* was more abundant in circumneutral to slightly alkaline waters, and reached its highest abundance in the surface sediments of Clear Pond ($pH = 7.2$).

The earliest date at which the shift in Mallomonadaceae could have occurred was approximately 60–70 years ago. This estimate is made by assuming a constant sedimentation rate (~ 25 years cm^{-1}) between the rise in non-arboreal pollen at the 6 cm level (~ 1850) and 1979, the year the core was taken. However, the sediment was less dense at the top of the core, so that accumulation rates must have been greater near the sediment surface, and the shift in Mallomonadaceae undoubtedly occurred more recently. Nevertheless, the acidification began at least 30 years ago, as the earliest limnological data available for Deep Lake are from 1954 when the pH was already recorded as low as 4.7¹¹. This is consistent with fisheries data cited previously, since it is likely that the decline in lake pH preceded the decline in fish populations. The inferred acidification probably resulted in large part from the increased atmospheric deposition of strong acids.

Our interpretation of Deep Lake's recent history is further substantiated by the stratigraphic distribution of diatoms. Before the proposed period of acidification, the diatom flora consisted of a diverse assemblage of indifferent to acidophilous taxa [for example *Frustulia rhomboides* (Ehr.) De T., *Anomoeoneis serians* var. *brachysira* (Bréb. ex Kütz.) Hust.]. However, in the surface sediment, the assemblage is dominated (56%) by an as yet undescribed taxon [*Fragilaria* cf. *virescens* var. 1.]. Based on Charles' diatom survey of the Adirondack lakes⁹, this new *Fragilaria* is an acidobiontic form, reaching its highest abundance in lakes with a $pH < 5.0$.

Our data indicate that the Mallomonadaceae are sensitive to changes in pH , and can therefore provide important palaeoecological data on lake acidification. Because sediment preparations for mallomonadacean scales and diatoms are identical, scales can easily be incorporated into palaeolimnological studies where diatoms are being analysed. In contrast to euplanktonic diatoms, which appear to be excluded in waters with $pH < 5.8$ – 6.0^9 , several mallomonadacean taxa thrive in acidic lakes. Hence, mallomonadacean scales probably represent the only algal microfossils that will reflect the euplankton in acidic lakes. Palaeolimnological studies that utilize both diatom frustules and mallomonadacean scales should provide a more accurate reconstruction of lake history.

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1. Takahashi, E. *Electron Microscopical Studies of the Synuraceae (Chrysophyceae) in Japan* (Tokai University Press, 1978).
2. Smol, J. P. *Can. J. Bot.* **58**, 458–465 (1980).
3. Munch, C. S. *Freshwat. Biol.* **10**, 61–66 (1980).
4. Battarbee, R. W., Cronberg, G. & Lowry, S. *Hydrobiologia* **71**, 225–232 (1980).
5. Smol, J. P., Brown, S. R., & McNeely, R. N. *Hydrobiologia* **103**, 125–130 (1983).
6. Smol, J. P., Charles, D. F., & Whitehead, D. R. *Can. J. Bot.* (in the press).
7. Schofield, C. L. *Acidification of Adirondack Lakes by Atmospheric Precipitation: Final Report, Project F-28-R* (New York State Department of Environmental Conservation, Albany, 1976).
8. Pfeiffer, M. H. & Festa, P. J. *Acidity Status of Lakes in the Adirondack Region of New York in Relation to Fish Resources* (New York State Department of Environmental Conservation, Albany, 1980).
9. Charles, D. F. thesis, Indiana Univ. (1982).
10. Cushing, E. J. & Wright, H. E. *Jr Ecology* **46**, 380–384 (1965).
11. New York State Department of Environmental Conservation Lake and Pond Survey records, Region 5, Ray Brook, New York, USA.
12. Driscoll, C. T., Jr, Baker, J. P., Bisogni, J. J., Jr, & Schofield, C. L. *Nature* **284**, 161–164 (1980).
13. Cronberg, G. & Kristiansen, J. *Bot. Notiser* **133**, 595–618 (1980).
14. Nicholls, K. *Nova Hedw.* **34**, 88–124 (1982).
15. Kling, H. & Kristiansen, J. *Nord. J. Bot.* **3**, 269–290 (1983).

Photodriven charge separation in a carotenoporphyrin–quinone triad

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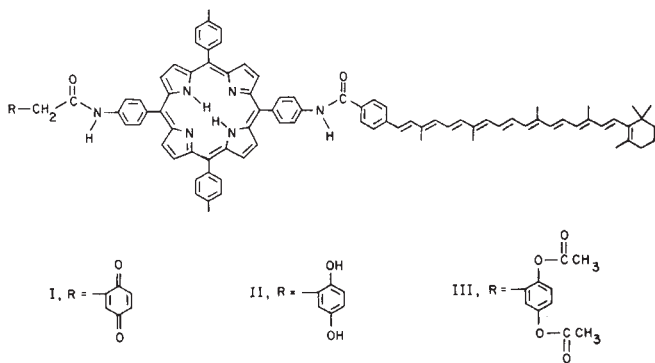
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The key steps in the photosynthetic conversion of light to chemical potential energy include not only photodriven charge separation, but also prevention of the back-reaction (charge recombination). Although the first of these steps has been achieved in several biomimetic solar energy conversion systems, retarding the back-reaction has proved more difficult. This may be accomplished by rapidly moving the electron, the hole, or both away from the site of excitation to more stabilizing environments. In photosynthetic membranes, the electron is transferred sequentially over several closely coupled molecules, including tetrapyrroles and quinones^{1–3}. In semiconductor/liquid interfacial systems both the electron and the hole migrate following excitation^{4,5}. We now report that substantial slowing of the back-reaction has been achieved with a tripartite molecule in which a long-lived photodriven charge-separated state of relatively high potential is formed from an excited singlet state in accordance with the above principles. This molecular triad (compound I) consists of a tetraarylporphyrin covalently linked to both a carotenoid and a quinone. In solution, excitation of the porphyrin moiety by visible light results in the rapid (< 100 ps) formation of a transient species $C^{+}-P-Q^{-}$, with a lifetime on the μs time scale and an energy more than 1 eV above the ground state.

Compound I was synthesized using methods previously developed for carotenoporphyrins^{6–10}. The 7'-apo-7'-(4-carboxyphenyl)- β -carotene was prepared by the Wittig reaction of the ylide of 4-carbomethoxybenzyltriphenylphosphonium bromide with β -apo-8'-carotenal, followed by saponification of the resulting methyl ester. The 5,15-bis(4-aminophenyl)-10,20-bis(4-methylphenyl)porphyrin was prepared by reduction of the corresponding dinitro porphyrin, and the carotenoid was linked to this porphyrin using dicyclohexylcarbodiimide and 4-dimethylaminopyridine. Coupling of this carotenoporphyrin to 2,5-diacetoxyphenylacetic acid yielded compound III, which was deacetylated with potassium borohydride to generate hydroquinone II. The oxidation of II to I was achieved with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone or lead oxide. The fast atom bombardment (FAB) mass spectrum of III yielded a monoisotopic mass ($M+1$) of 1,423.5 (calculated for $C_{96}H_{90}N_6O_6$; 1,423.7).

The 500 MHz 1H -NMR spectra of I, II and III in $CDCl_3$ solution support the assigned structures and also yield conformational information. The aromatic, 7' and 8' proton resonances of the carotenoid portions of the molecules are shifted downfield from their positions in the corresponding carotenoid aniline amide, whereas the chemical shifts of the remaining carotenoid protons are virtually unchanged. These downfield shifts (≤ 0.16 p.p.m.) indicate that the carotenoid is in the deshielding region of the porphyrin ring current field. Quantitative calculations¹¹ for III show that the carotenoid chain lies approximately in the plane of the porphyrin and is extended away from the



porphyrin π -electron system (Fig. 1). The protected hydroquinone side chain is also in an extended, rather than folded, conformation. Ring current calculations yield the conformation shown in Fig. 1, and are consistent with rapid rotational jumps of the $-\text{CH}_2$ -diacetoxyphenyl residue among the various equilibrium positions about the CH_2 -CO bond, only one of which is shown. Conversion of III to II or I results in small changes in chemical shifts for the protons of the quinone side chain, as expected, and has very little effect ($\Delta\delta \approx 0.05$ p.p.m.) on the porphyrin or carotenoid proton resonances. Thus, it is reasonable to conclude that the time average solution conformation of each of these triads is also extended with the carotenoid and quinone moieties rather widely separated.

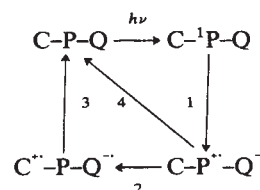
Nanosecond flash excitation¹² at 600 nm of a solution of compound I in CH_2Cl_2 resulted in the transient absorption spectrum shown in Fig. 2 with $\lambda_{\text{max}} = 970$ nm and $t_{1/2} \sim 170$ ns. This signal was not observed with II or III. Figure 2 also shows the transient absorption after pulse radiolysis of III and of a model for its carotenoid component. The assignment of the pulse radiolysis¹³ spectrum, and therefore the flash photolysis spectrum, to a carotenoid cation radical is straightforward¹⁴⁻¹⁷. A carotenoid ground state bleaching signal ($\lambda_{\text{max}} \sim 460$ nm, $t_{1/2} \sim 170$ ns) appeared concomitantly with the cation radical absorption. (A small, long-lived bleaching component ascribed to a carotenoid triplet arising from trace amounts of II was also observed.) Saturation of the CH_2Cl_2 solution with tetra-*n*-butylammonium tetrafluoroborate increased the yield of carotenoid cation radical by a factor of at least six, increased $t_{1/2}$ to ~ 2.5 μs , and blueshifted the absorption maximum to ~ 940 nm. In acetonitrile solution $\lambda_{\text{max}} = 910$ nm and $t_{1/2}$ was ~ 3 μs .

Picosecond techniques were used to measure the rise of the cation radical (Fig. 3). An absorbance increase was observed at 950 nm and assigned to the carotenoid cation radical, as confirmed by a spectrum taken at 500 ps. The rise of the cation radical occurs within ~ 100 ps, and is delayed by ~ 30 ps with

respect to the response time of the apparatus as determined by the photobleaching of pinacyanol chloride¹⁸. At 600 nm, an absorbance increase appears with the response time of the instrument and decays within 100 ps to a small residual absorption. This absorbance increase may be assigned to the porphyrin first excited singlet state (^1P) and/or an intermediate porphyrin cation radical (see below)^{19,20}.

The quantum yield of carotenoid cation radical was ~ 0.25 in CH_2Cl_2 solution saturated with tetra-*n*-butylammonium tetrafluoroborate. This measurement was made by a comparative method²¹ using free base *meso*-tetra-*p*-tolylporphyrin in benzene as the standard ($\epsilon_{441} (^3\text{porphyrin}) = 6.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (ref. 8) and $\phi (^3\text{porphyrin}) = 0.9$ (ref. 22)). An ϵ_{970} (carotenoid cation radical) $\sim 1.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was determined from the ratio of δA_{460} (the bleaching component having $t_{1/2} \sim 170$ ns) to $\delta A_{970} = 1:2.7$ and assuming¹⁷ ϵ_{460} (carotenoid cation radical) $= 0.5 \epsilon_{460}$ (carotenoid S_0)¹⁵⁻¹⁷. In pure CH_2Cl_2 , the quantum yield was ~ 0.04 ; similar values were found in acetonitrile and acetone.

The simplest scheme that is consistent with the spectroscopic observations is shown below



(minor pathways, triplet states and spin multiplicities of the ionic states are ignored). By analogy with simple porphyrin-quinone systems^{19,20,23-25}, it is reasonable to assume that the dominant relaxation pathway (1) from $\text{C-}^1\text{P-Q}$ is quenching of ^1P via electron transfer to yield $\text{C-P}^{\cdot-}\text{-Q}^{\cdot-}$. The immediate rise of the 600-nm absorption on excitation (Fig. 3) is consistent with the formation of $\text{C-}^1\text{P-Q}$ and its rapid conversion to $\text{C-P}^{\cdot-}\text{-Q}^{\cdot-}$. (The relative contributions of ^1P and $\text{P}^{\cdot-}$ to this

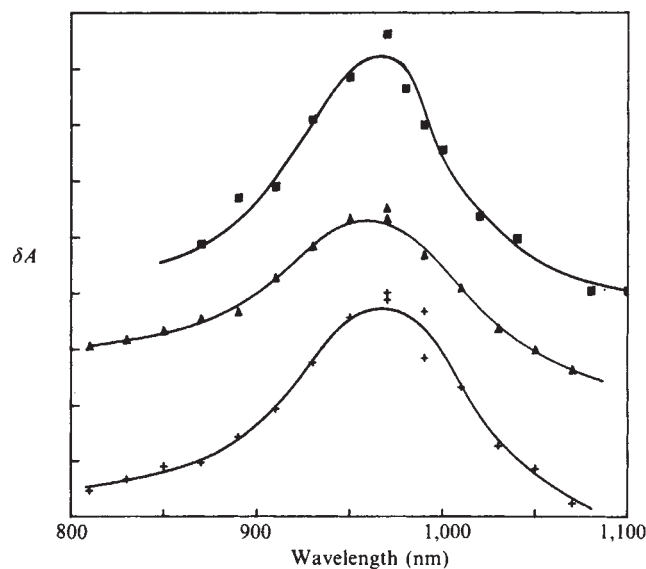


Fig. 2 Transient absorption spectra of carotenoid cation radicals. ■, Absorption spectrum immediately after 10-ns 600-nm flash excitation¹² of 10^{-5} M compound I in CH_2Cl_2 . ▲, Absorption spectrum 2 μs after pulse radiolysis of argon-flushed $\sim 5 \times 10^{-5}$ M compound III in CH_2Cl_2 . +, Absorption spectrum 4 μs after pulse radiolysis of argon-flushed 10^{-5} M aniline amide of 7'-apo-7'(4-carboxyphenyl)- β -carotene in CH_2Cl_2 . For the pulse radiolysis experiments, radiation doses (~ 20 Gy per 50 ns pulse) were provided by a 12-MeV Vickers electron linear accelerator¹³. Quartz capillary cells of 2 cm optical path were used. The δA scales for the three spectra are different, and baselines have been offset for clarity.

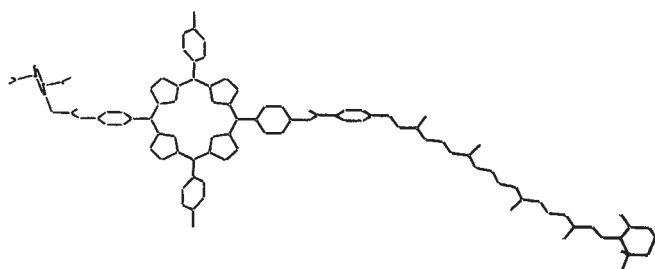


Fig. 1 Time averaged solution conformation of triad III as calculated from porphyrin ring current-induced $^1\text{H-NMR}$ chemical shift changes. The hydrogen atoms are not shown. The acetylated hydroquinone moiety is seen edge-on at the left, whereas the carotenoid extends to the right. In this perspective, the porphyrin is not coplanar with the plane of the figure. The chemical shifts of the acetyl methyl protons are consistent with rapid reorientation by π radians about the bonds linking the acetoxy oxygens to the aromatic ring.

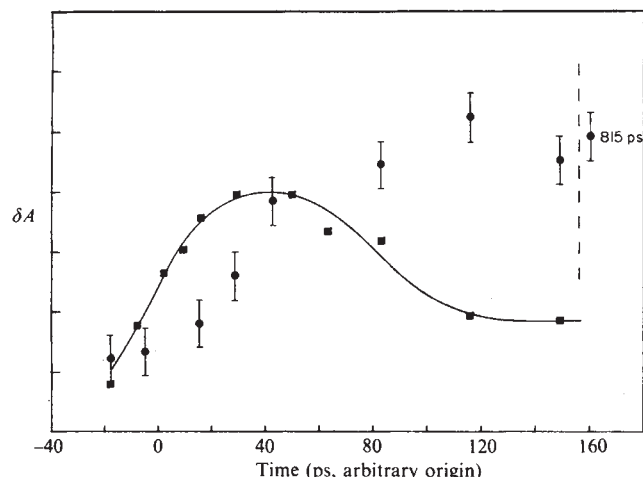


Fig. 3 Transient absorption kinetics for compound I (10^{-4} M) in CH_2Cl_2 solution saturated with tetra-*n*-butylammonium tetrafluoroborate after 532 nm pulse excitation (27 ps FWHM)³². The probe light was the ps continuum of light, analysed after the sample cell (1 mm path length) using Pomfret interference filters (3 nm bandwidth) centred at 600 nm and at 950 nm ($\delta A = 0.08$ per division). Each point is an average of four laser shots. The circles with error bars represent the response at 950 nm (carotenoid cation radical), whereas the squares give δA at 600 nm. The error bars at 600 nm are comparable with those at 950 nm, but have been omitted for clarity.

absorption are not resolved.) The decay of this absorption and concurrent rise of the carotenoid cation radical absorption reflect the formation of $\text{C}^{\bullet+}\text{-P-Q}^{\bullet-}$ (step 2). The dramatic shortening of the porphyrin first excited singlet lifetime ($\tau \sim 10$ ns for a typical porphyrin) suggests that the species $\text{C-P}^{\bullet+}\text{-Q}^{\bullet-}$ is formed with a quantum yield approaching unity. Furthermore, the rapid decay of ^1P and rise of carotenoid cation radical rule out the participation of the porphyrin triplet in the primary electron transfer process. In addition, there is no evidence for direct excitation of Mulliken-type²⁶ porphyrin-quinone charge transfer states, because the absorption spectra at 400–800 nm for I, II and III are identical to within 1%.

The next step in the formation of the final charge-separated state is the electron transfer process $\text{C-P}^{\bullet+}\text{-Q}^{\bullet-} \rightarrow \text{C}^{\bullet+}\text{-P-Q}^{\bullet-}$ (step 2) which competes with the recombination reaction (step 4). In pure CH_2Cl_2 the yield of (2) is $\sim 4\%$, whereas at high salt concentration it increases markedly. This observation suggests that the outcome of the competition between (2) and (4) is strongly dependent on the ionic environment. It seems certain that the redox potential of the final state $\text{C}^{\bullet+}\text{-P-Q}^{\bullet-}$ is > 1 V, based on $E_{1/2}$ values for related unlinked molecules^{27–29}. Thus, a substantial fraction of the 1.8-eV porphyrin lowest excited singlet state has been conserved as chemical potential.

As was found with certain other carotenoporphyrins^{7,8}, the triads mimic antenna and photoprotective processes observed in photosynthetic membranes. In compounds II and III, singlet energy transfer ($^1\text{C-P-Q} \rightarrow \text{C-}^1\text{P-Q}$, antenna function) is $\sim 10\%$ efficient. As is the case in the natural system, triplet states are formed in much greater yield when the primary acceptor is reduced (II or III) before excitation. Quenching of the porphyrin triplet state in III ($\text{C-}^3\text{P-Q} \rightarrow \text{C-}^3\text{P-Q}$) occurs in less than 20 ns, ensuring essentially complete photoprotection from singlet oxygen (a detailed report of the singlet and triplet energy transfer processes in the triads will be presented elsewhere).

The observation of a carotenoid cation radical in I has biological relevance in that the formation of a carotenoid cation radical has been observed in photosystem II in certain conditions²⁷. Note also that the formation of a carotenoid radical cation in I in organic solvents suggests that charge conduction by a carotenoid through media of low dielectric constant³⁰ is possible. Furthermore, the observation that an ionic environment pro-

motes the formation of $\text{C}^{\bullet+}\text{-P-Q}^{\bullet-}$ is consistent with the suggestion that specific charged sites on proteins at the reaction centre in photosynthetic membranes may be important in mediating charge separation³¹.

The formation of a long-lived charge-separated state from the first excited singlet state of this molecular triad with a reasonable quantum yield has significance for the design of artificial photosynthetic systems. The key to obtaining long lifetimes of the charge-separated state appears to be the interposition of a neutral porphyrin between the widely separated ions, coupled with energetics which are unfavourable for recombination via species such as $\text{C-P}^{\bullet+}\text{-Q}^{\bullet-}$ or $\text{C}^{\bullet+}\text{-P-Q}$. Moreover, note that I is relatively stable; there is little change in the sample even after hundreds of laser flashes over several days.

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- Dutton, P. L., Prince, R. C. & Tiede, D. M. *Photochem. Photobiol.* **28**, 939–949 (1978).
- Sauer, K. *Acc. chem. Res.* **11**, 257–264 (1978).
- Mathis, P. & Paillotin, G. in *The Biochemistry of Plants* Vol. 8 (eds Hatch, M. D. & Boardman, N. K.) 97–161 (Academic, New York, 1981).
- Gerischer, H. in *Topics appl. Phys.* **31**, 115–172 (1979).
- Wrighton, M. S. *Acc. chem. Res.* **12**, 303–310 (1979).
- Dirks, G., Moore, A. L., Moore, T. A. & Gust, D. *Photochem. Photobiol.* **32**, 277–280 (1980).
- Moore, A. L., Dirks, G., Gust, D. & Moore, T. A. *Photochem. Photobiol.* **32**, 691–695 (1980).
- Bensasson, R. V. *et al. Nature* **290**, 329–332 (1981).
- Moore, A. L. *et al. Science* **216**, 982–984 (1982).
- Liddell, P. A. *et al. Photochem. Photobiol.* **36**, 641–645 (1982).
- Chachaty, C. *et al. Org. magn. Reson.* (in the press).
- van Best, J. A. & Mathis, P. *Rev. scient. Instrum.* **49**, 1332–1335 (1978).
- Keene, J. P. *J. scient. Instrum.* **41**, 493–496 (1964).
- Dorfman, L. M., Sujdak, R. J. & Bockrath, B. *Acc. chem. Res.* **9**, 352–357 (1976).
- Mathis, P. & Vermiglio, A. *Photochem. Photobiol.* **15**, 157–164 (1972).
- Dawe, E. A. & Land, E. J. *JCS Faraday Trans. 1* **71**, 2162–2169 (1975).
- Chauvet, J.-P., Viogy, R., Land, E. J., Santus, R. & Truscott, T. G. *J. phys. Chem.* **87**, 592–601 (1983).
- Mialocq, J. C. *Chem. Phys.* **73**, 107–115 (1982).
- Migita, M. *et al. Chem. Phys. Lett.* **84**, 263–266 (1981).
- Bergkamp, M. A., Dalton, J. & Netzel, T. L. *J. Am. chem. Soc.* **104**, 253–259 (1982).
- Bensasson, R. & Land, E. J. *Photochem. photobiol. Rev.* **3**, 163–191 (1978).
- Moore, T. A., Benin, D. & Tom, R. J. *J. Am. chem. Soc.* **104**, 7356–7357 (1982).
- Kong, J. L. Y., Spears, K. G. & Loach, P. A. *Photochem. Photobiol.* **35**, 545–553 (1982).
- Tabushi, I., Koga, N. & Yanagita, M. *Tetrahedron Lett.* 257–260 (1979).
- Ho, T. F., McIntosh, A. R. & Bolton, J. R. *Nature* **286**, 254–256 (1980).
- Mulliken, R. S. *J. Am. chem. Soc.* **72**, 600–608 (1950); **74**, 811–824 (1952).
- Schenck, C. C., Diner, B., Mathis, P. & Satoh, K. *Biochim. biophys. Acta* **680**, 216–227 (1982).
- Paramonova, L. I., Naush, Ya., Kreslavskii, V. D. & Stolovitskii, Yu. M. *Biophysics* **27**, 199–203 (1982).
- Prince, R. C., Gunner, M. R. & Dutton, P. L. in *Function of Quinones in Energy Conserving Systems* (ed. Trumpower, B. L.) 29–33 (Academic, New York, 1982).
- Platt, J. R. *Science* **129**, 372–374 (1959).
- Mauzerall, D. *Israel J. Chem.* **21**, 321–324 (1981).
- Mialocq, J. C., Amouyal, E., Bernas, A. & Grand, D. *J. phys. Chem.* **86**, 3173–3177 (1982).

Sensitivity for vertical retinal image differences

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The detection of horizontal disparities between the right and left retinal images is accepted as an important component in the visual perception of depth. It has occasionally been pointed out^{1–3} that a disparity will also occur in the vertical when a target is nearer to one eye than the other and could be used as a second component³. Here it is shown that while it is possible to detect size differences between the retinal images in the vertical direc-

tion, the sensitivity is at least one order of magnitude less than the horizontal differences. It had been generally accepted that vertical disparities cannot be detected (literature summarized by Tschermak⁴), but there are clear reports of the ability of subjects to respond to relative differences in the vertical magnification of the images of the two eyes, the so-called induced size effect². It manifests itself in the appearance of a visual scene as if there had been an opposite horizontal magnification and is, therefore, in the correct direction if it is to be utilized in asymmetrical convergence.

Two reasons make it opportune to examine this matter experimentally. Mayhew and Longuet-Higgins³ have questioned the applicability of recent findings^{5,6} which have again failed to show any significant sensitivity of normal observers to imposed vertical disparities in their retinal images. They point out that in actual viewing situations where a target is nearer to one eye than the other, as indeed also in the more artificial conditions for observing the induced size effect, the vertical disparities are appropriately distributed throughout the visual field in a plausible manner. Tests for detection of vertical disparity in a simple unitary target may, therefore, not be successful. The second reason for looking into this matter with some care is the extraordinary sensitivity of most subjects to horizontal disparities. Thresholds of a few seconds of arc indicate that a complex apparatus has to be present to extract this information from the retinal images. Neurophysiological studies of early cortical stages in the mammal indicate disparity coding with at least one and possibly two orders of magnitude less sensitivity⁷ and, significantly, the scatter of disparities is about the same in the horizontal and the vertical direction^{8,9}. One would thus like to know whether the mechanism for distilling the cortical cells' coarse disparity coding into the fine disparity signals demonstrated in stereoacuity is needed not only in the horizontal direction, but also vertically as would be the case if subjects indeed had good ability to detect vertical disparities, no matter how this ability actually manifested itself.

In the experiments a compromise was struck between the abstract, single feature patterns used in previous disparity detection research and the more variegated visual configurations that seem to be needed to elicit the induced size effect². Five small squares with sides of 5 arc min were seen by both eyes at a distance of 3 m. They were laid out in an inverted V, the middle two squares being 30 arc min apart horizontally and the lower pair 1° apart (Fig. 1). This pattern was chosen because any implicit horizontal disparity induced by vertical magnification⁶ would not manifest itself in a tilt of the fronto-parallel plane around a vertical axis, which is the essence of the induced size effect. The rest of the visual field was entirely dark. Because the induced size effect is said to take some time to develop, exposure duration was 1 s, several times longer than needed for good horizontal disparity discrimination.

During an experimental session, the pattern was presented every 3 s and good fixation was maintained in the intervals by showing a fronto-parallel set of four brackets with a central cross, all dimensions being equal in the two eyes. By means of polaroids and a half-silvered mirror it was arranged that one eye saw the display on one oscilloscope and the other eye on another. During each stimulus presentation, the vertical gain of one scope relative to that of the other was set to a value chosen at random from an ensemble consisting of seven gains, for example, 0.94, 0.96, 0.98, 1.00, 1.02, 1.04 and 1.06. That is to say, the whole pattern seen by one eye was 6%, 4%, 2% or 0% larger or smaller vertically than that of the other, randomly chosen for a given presentation. The subject's response was restricted to the decision whether the little squares in the right or left limb of the inverted V configuration were nearer to him. Results (Fig. 1, left), accumulated in several sessions of 300 responses, were fitted by the methods of probits, which enables the identification of a threshold, that is, that image magnification at which the subject on 75% of occasions saw the pattern rotated out of the fronto-parallel plane around a vertical axis in the indicated direction. Thresholds are summarized in Table I for three experienced stereoscopic subjects. Fragmentary results in a fourth subject gave concordant findings. Also shown are the results for equivalent runs in which the magnification changes were given in the horizontal direction, where the situation accurately mimics an actual rotation of the fronto-parallel plane around a vertical axis. Each pair of conditions (horizontal and vertical) is based on a total of 2,000 responses, obtained over several days under strictly comparable conditions.

The findings summarized in Table 1 allow conclusions about both of the questions raised. First, the sensitivity for discrimination of vertical magnification differences in the two eyes is 14, 12 and 3.5 times lower in the three subjects, respectively, than that for horizontal magnification differences. Second, it seems indeed possible to show a small induced effect with appropriate kinds of abstract stimuli: complex patterns, long exposure durations and a relatively conflict-free visual environment seem necessary. Vertical magnification of one eye then produces a restructuring of visual space as if the other eye had been subjected to horizontal magnification. The experiment reported here is, however, not an exact replication of the standard demonstration of the induced size effect², where an ink-splattered glass pane is set to appear fronto-parallel by a subject wearing a vertically magnifying lens over one eye.

Succinctly stated, our three subjects viewing a fronto-parallel pattern with a 3% horizontal magnification would call it tilted towards their left on 98%, 99% and 78% of all presentations, respectively; of the presentations with 3% vertical magnification, 58%, 60% and 61% respectively would be called tilted towards their right. The percentages each have a standard error of no more than 3% and even in the second set they are clearly

Fig. 1 *a*, Results of 2,000 forced-choice responses from subject G.W., without error feedback, in which right and left retinal images of the pattern seen binocularly (shown in inset) were presented at random with one of the indicated magnification ratios, either horizontally or vertically. *b*, Results of a similar experiment where the pattern elements remained of constant size throughout, but were given differential horizontal or vertical displacements in the two eyes to produce binocular disparities. Lines are best fit probits and allow accurate threshold quantitation: half the image differences for which 75% of the responses are at criterion. Sensitivity for vertical image size differences, whether generated by magnification (*a*) or disparity (*b*), is about an order of magnitude less than for horizontal ones and the responses are of opposite polarity.

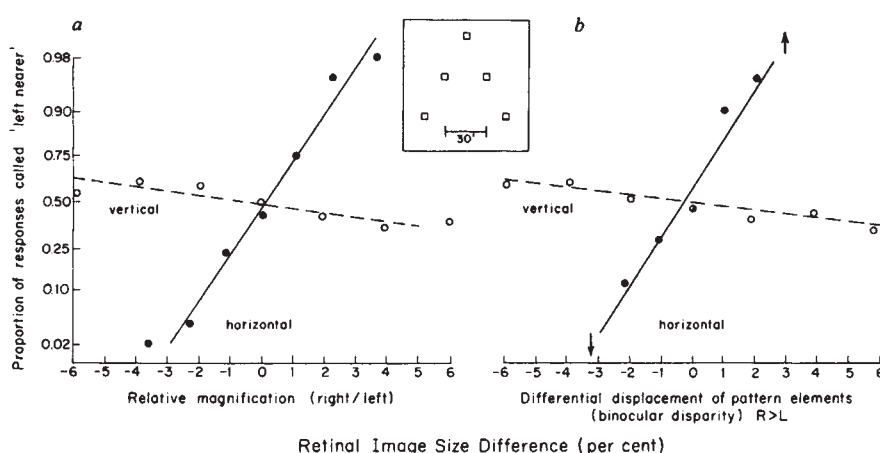


Table 1 Threshold difference between subject's right and left retinal images at which he could detect an apparent rotation of a fronto-parallel plane around a vertical axis

Direction	Subject		
	GW	MG	RY
Magnification			
Horizontal	1.1+0.06	0.9+0.05	2.9+0.2
Vertical*	-12.9+2.3	-12.7+2.7	-10.1+1.8
Disparity			
Horizontal	0.9+0.06	0.9+0.06	1.5+0.11
Vertical*	-15.5+2.6	-16.9+3.0	-29.3+10.2

Magnification: whole pattern was magnified in the indicated direction—threshold is expressed as per cent magnification (right/left) retinal images. Disparity: pattern elements remained of same size but were differentially displaced in the two eyes to give binocular disparity in the indicated direction, threshold is expressed as per cent difference in target separations in the two eyes.

* The minus sign signifies that the apparent fronto-parallel plane appeared nearer at the subject's right when the retinal image of the right eye was larger vertically than that of the left.

statistically different from random, which would give a 50% response. In a related experiment using gratings with spatial frequency differing in the two eyes, Rigaudiere¹⁰ observed much less tilt of the apparent fronto-parallel plane for horizontal than for vertical patterns.

A large difference between horizontal and vertical also showed up when the experiments were repeated with the same geometrical target and exposure duration, but with the depth clue given by pure disparity and not magnification. In practice this meant that the squares making up the target were of identical size in the two eyes under all stimulus conditions, but their relative placements on the two retinas was dictated solely by disparity considerations. This could, however, still be expressed in percentage terms, although now referring to the relative separation of identically sized target elements on the two retinas. The results (Fig. 1, right, and Table 4) confirm the earlier observations of massive differences in sensitivity for horizontal versus vertical disparities⁵. The ratio is about 20 for all three observers.

An induced size effect, therefore, exists not only in the situation used by Ogle, but even here where the experimental situation is governed by the kind of stimulus abstraction commonly used in the psychophysical analysis of the substrates of space perception. The particular patterns in the experiment described here preclude the direct use of implicit horizontal disparity information contained in stimuli with uniaxial vertical magnification. This cannot, therefore, be the sole clue for the induced size effect, as has sometimes been thought^{5,6}.

To see whether there are simple ways of enhancing vertical disparity detection beyond the levels shown in the experiments reported above, two additional factors have been cursorily examined, longer exposure durations and increased pattern size. There was no important change in the threshold when the target was presented for 5 s; and a target consisting of a 7×7 sheet of dots extending over the central 3° of the visual field still did not suffice to lower the disparity threshold materially, so it would appear necessary to postulate spatial integration over a much larger portion of the visual field. If this is the case, we have yet another variable that sets off vertical from horizontal disparity detection, because stereoacuity can be a few seconds of arc for just two small squares separated by 10–20 arc min.

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- Householder, A. S. *Bull. Math. Biophys.* **5**, 155–160 (1943).
- Ogle, K. N. *Binocular Vision* (Saunders, Philadelphia, 1950).

- Mayhew, J. E. W. & Longuet-Higgins, H. C. *Nature* **297**, 376–378 (1982).
- Tschermak, A. *Handbuch der normalen und pathologischen Physiologie* Vol. XII/2 (Springer, Berlin, 1932).
- Westheimer, G. *Invest. ophthalm. vis. Sci.* **17**, 545–551 (1978).
- Arditi, A., Kaufman, L. & Movshon, J. A. *Vision Res.* **21**, 755–764 (1981).
- Poggio, G. F. & Talbot, W. H. *J. Physiol., Lond.* **315**, 469–492 (1981).
- Joshua, D. E. & Bishop, P. O. *Expl Brain Res.* **10**, 389–416 (1970).
- Ferster, D. *J. Physiol., Lond.* **311**, 623–655 (1981).
- Rigaudiere, F. *Vision Res.* **15**, 931–938 (1975).

Interspecific chimaerism between sheep and goat

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In rodents, chimaeric blastocysts produced by combining embryonic cells of two different species have been used in investigations of cell lineage and interaction during development (*Mus musculus*–*Rattus norvegicus*^{1,2}, *M. musculus*–*Clethrionomys glareolus*³, *M. musculus*–*Mus caroli*⁴). However, interspecific chimaerism also offers new approaches to the study of reproductive incompatibilities between species and may even allow such incompatibilities to be neutralized, thus improving the chances of successful hybridization and interspecific embryo transplantation⁵. We report here the production of sheep–goat chimaeras by embryo manipulation and the use of interspecific chimaerism to allow successful interspecific embryo transplantation in sheep and goats.

So far, the *M. musculus*–*M. caroli* chimaeras described by Rossant *et al.*^{4–6} seem to be the only viable interspecific mammalian chimaeras to have been produced by embryo manipulation. The two species concerned are closely related and, although they do not normally interbreed, they can be hybridized⁷. The domestic sheep (*Ovis aries*, 2n=54) and the domestic goat (*Capra hircus*, 2n=60) are less closely related^{9,10} but neither a sheep nor a goat has carried a live hybrid fetus to term in a controlled experiment except when special procedures were used to reduce interspecific incompatibility¹¹. The rationale of the latter procedures as well as the reproducibility of the results have been questioned¹². Experimental hybrid pregnancies in both sheep and goat females have often proceeded beyond the time of implantation but, invariably, by the end of the second month the fetus has died^{12–14}. The same applies to pregnancies resulting from transfer of sheep embryos to goat recipients, while goat embryos transferred to sheep recipients have not survived beyond the first month^{13,15}. The transfer of an embryo belonging to the same species as the recipient together with the foreign embryo does not seem to alter the survival prospects of the latter¹⁵. In all the above categories of interspecific pregnancies, placental abnormalities, possibly due to maternal immunological reaction against foreign antigens of the conceptus, have been the immediate cause of abortion^{16–19}.

The present study comprises three series of experiments. In the first series, single blastomeres from 4-cell goat embryos were combined with single blastomeres from 4-cell sheep embryos (Table 1, expt 1a), or with single blastomeres from 8-cell sheep embryos (expt 1b) in evacuated zonae pellucidae as described by Willadsen²⁰. Up to four interspecific embryos of identical composition were made from the same two parent embryos. In the second series of experiments, interspecific 'giant' embryos were produced either by surrounding an 8-cell goat embryo from which the zona pellucida had been removed with the dissociated blastomeres of three 8-cell sheep embryos (expt 2a) or by surrounding a similarly denuded 8-cell sheep embryo with the separated blastomeres of three 8-cell goat embryos.

Table 1 The development of chimaeric embryos composed of sheep and goat cells

Experiment	1		2		3	
	a	b	a	b	a	b
Composition of embryos*	$\frac{1}{4}S + \frac{1}{4}G$	$\frac{1}{8}S + \frac{1}{4}G$	$\frac{8}{8}G + 3 \times \frac{8}{8}S$	$\frac{8}{8}S + 3 \times \frac{8}{8}G$	ICMG + BLS	ICMS + BLG
No. cultured in temporary recipients	31	4	4	3		
No. forming interspecific blastocysts	17	2	4	3		
Species of definitive recipients	S	G	S	G	S	G
No. transferred to definitive recipients	17	2	4	3	11	3
No. of full-term young	7	2	3	2	9	3
No. of lambs	4	2(?)	1†	0	6	1
No. of kids	0	0	0	1†	1	2
No. of interspecific chimaeras	3	0	2	1	2	0

* As regards embryo composition, $\frac{1}{4}$ represents one blastomere from a 4-cell embryo; $\frac{1}{8}$ represents one blastomere from an 8-cell embryo; and $\frac{8}{8}$ represents all eight blastomeres of an 8-cell embryo. BL, day 8 blastocyst; ICM, inner cell mass and polar trophectoderm from day 8 blastocyst (S, sheep and G, goat).

† Intraspecific chimaera.

The composite embryos in expts 1 and 2 were embedded in agar and cultured in ligated sheep oviducts for 5 (expt 1) or 4 days (expt 2)²⁰. They were then examined as fresh specimens (Table 1) and those which had developed into normally organized chimaeric blastocysts were transferred to recipient ewes (expt 1a and 2a) or recipient goats (expt 1b and 2b), on day 7 of the oestrous cycle (day of onset of oestrus = day 0).

In a third series of experiments, the inner cell mass and polar trophectoderm from day 8 goat blastocysts were inserted into day 8 sheep blastocysts by the technique described by Gardner²¹ (expt 3a). In the same way, sheep inner cell masses and polar trophectoderm were combined with goat blastocysts (expt 3b). The blastocysts were transferred to sheep (expt 3a) or goat recipients (expt 3b) on day 8 of their oestrous cycle.

The main results of the experiments are presented in Table 1. The proportion of composite embryos which formed normally organized blastocysts during culture *in vivo* was somewhat lower than that observed in parallel experiments on intraspecific chimaerism in sheep, but judged by the initial pregnancy rate obtained after transplantation of interspecific blastocysts, they were as viable as intraspecific embryos of the same cell number, whether chimaeric or non-chimaeric. However, one pregnancy

failed at about day 60, and two fetuses were aborted near term in expt 1a.

The seven full-term young (and the two aborted fetuses) in expt 1a all had the general appearance of lambs, but in three of these animals the fleece had transverse bands and patches of hair contrasting sharply with the surrounding densely curled wool (Figs 1, 2). Two of these animals each had a twin, produced from the same two parent embryos. Both of the respective twins were lambs with ordinary fleeces. The hairy bands were therefore thought to represent goat tissue, and the three animals were judged to be overt sheep-goat chimaeras (Fig. 3). The two young in expt 1b both looked like ordinary lambs; both were stillborn. The three young produced in expt 2a also had the general appearance of lambs, but two of these had hairy bands and patches in their fleeces similar to those observed in three of the lambs in expt 1a. These two lambs, which were dead at delivery, were therefore also judged to be overt sheep-goat chimaeras. They had developed from a single 'giant' blastocyst. Both of the young produced in expt 2b were of the same general appearance as ordinary kids. However, in one of them (Fig. 4) the normal straight goat hair was replaced by densely curled wool in symmetrically situated areas on the neck, shoulders and thighs. The wool was thought to be of sheep origin, and the animal was therefore judged to be an overt sheep-goat chimaera.



Fig. 1 Sheep-goat chimaera (standing) and its sheep twin (see expt 1a of Table 1). The two animals, both phenotypically male, developed from chimaeric embryos which shared both their sheep (Suffolk $\times$ Welsh Mountain) and their goat (Nubian $\times$ Saanen) parent embryo, and were, therefore, monozygotic twins with respect to their sheep component. Note the hairy bands and patches on the neck and trunk of the chimaera. Apart from the white patches on the legs (and ears) the pattern of pigmentation in the chimaera did not differ from that seen in its sheep twin, although the colours were brighter in the hairy areas than in the woolly areas.



Fig. 2 Dorsal view of another sheep-goat chimaera from expt 1a. Note the essentially asymmetric distribution of the relatively narrow hairy bands and patches along the midline. The type of fibre (wool or hair) produced in the epidermis is determined by the origin of the underlying dermis (sheep or goat) thus chimaerism, as observed at this stage, had its basis in mesodermally rather than ectodermally derived tissues, despite the absence of blood chimaerism. The pattern of distribution of the hairy bands is of somatic origin.



Fig. 3 Close-up of the back of a 1½ yr-old sheep-goat chimaera. By now the wool fibres have outgrown the hair fibres in the fleece so that the hairy bands and patches, although still clearly demarcated, are less conspicuous. Note that two types of fibres can be distinguished within the woolly areas: densely curled fibres of the type normally seen in Suffolk × Welsh Mountain sheep constitute the bulk of the fleece, but in addition there are tufts of longer, wavy (goat wool) fibres, which form a smaller proportion of the fleece but occur throughout. The presence of two types of wool fibres indicated chimaerism in ectodermally derived tissues in addition to the chimaerism in mesodermally derived tissues reflected in the presence of the hairy bands. The latter probably consist mainly of sheep hair but with tufts of goat hair interspersed.



Fig. 4 Sheep-goat chimaera from expt 2b at 1 yr old. In this animal many chimaeric features are evident, of which the strongly curved goat horns and the large patches of long, wavy (goat) wool are the most obvious. The distribution of wool, interesting by being essentially symmetric, coincides with areas of the epidermis overlying dermis derived from the lateral mesoderm. The animal, which contains both a male sheep and a male goat component, behaves like a male goat but has proved infertile in natural matings with female goats. The semen is of normal density, but almost all the spermatozoa have a characteristic tail defect.

Six of the nine young produced in expt 3a looked in every respect like ordinary lambs. Of the remaining three, one was similar to the chimaeric lambs in expt 1a, and another was similar to the chimaeric kid in expt 2b, except that its skin had transverse bands and patches of wool along the back; both of these animals were judged to be overt sheep-goat chimaeras. The last of the three animals produced in expt 3a looked in every respect like an ordinary kid. Two of the three young produced in expt 3b resembled ordinary kids, but the third, which was born as a twin to one of the kids, looked like a lamb.

Blood samples from those lambs and kids which were born alive were tested with respect to the following proteins which differ electrophoretically between sheep and goat²²: haemoglobin (adult and fetal), carbonic anhydrase, nucleoside phosphorylase, X-protein, malic enzyme, NADH diaphorase and transferrin. The only animal in which interspecific blood chimaerism was demonstrated was the overtly chimaeric kid in expt 2b, in which both parent species were represented in all blood proteins tested. The other overt chimaeras all had sheep-type blood proteins, while the remaining animals, including the kid in expt 3a and the lamb in expt 3b, had blood proteins entirely of the type corresponding to their species of physical appearance.

Thus, the present experiments show that sheep and goat blastomeres can form composite blastocysts and that such interspecific blastocysts are, in principle, viable and may give rise to animals which are sheep-goat chimaeras. In addition, the experiments have shown that a goat fetus can develop to term in a sheep and, similarly, that a sheep fetus can develop to term in a goat. In expt 1a the high abortion rate, the relatively low incidence of chimaeras among the offspring, and the disproportionately large contribution made by the sheep blastomeres even in those animals which were overt sheep-goat chimaeras, probably all reflect a reaction on the part of the recipient ewes against the goat component of the conceptus. Although intraembryonic incompatibility or competition between the two species may also have influenced the results, this is less likely to have been the principal cause of the predominance of the sheep component since extensive interspecific chimaerism was observed in one animal in expt 2 and in another in expt 3. The results of the latter experiment indicate that in sheep and goats it is possible to completely neutralize incompatibility between the conceptus and the recipient female by constructing the chimaeric embryo in such a way that the trophectoderm and hence the chorionic epithelium consists entirely of cells belonging to the same species as the recipient. It is probable that the low rate at which this was achieved reflects inadequacy of the particular approach used in that experiment. The results of expt 1b, although incomplete, suggest that a more economical and practical way of achieving the same objective is the use of asynchronous parent embryos and reduction of the total cell number to near the minimum required to form a normally organized blastocyst. This is in agreement with the observation made in experiments on intra-specific chimaerism in sheep (C.B.F. and S.M.W., unpublished observations).

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1. Stern, M. S. *Nature* **243**, 472-473 (1973).
2. Gardner, R. L. & Johnson, M. H. *Ciba Fdn Symp.* **29**, 183-200 (1975).
3. Mystkowska, E. T. *J. Embryol. exp. Morph.* **33**, 731-744 (1975).
4. Rossant, J. & Frels, W. I. *Science* **208**, 419-420 (1980).
5. Rossant, J., Croy, B. A., Chapman, V. M., Siracusa, L. & Clark, D. A. *J. Anim. Sci.* **55**, 1241-1248 (1982).
6. Rossant, J., Mauro, V. M. & Croy, B. A. *J. Embryol. exp. Morph.* **69**, 141-149 (1982).
7. West, J. D., Frels, W. I., Papaioannou, V. E., Karr, J. P. & Chapman, V. M. *J. Embryol. exp. Morph.* **41**, 233-243 (1977).
8. Gray, A. P. *Mammalian Hybrids*, 130-132 (Commonwealth Agricultural Bureaux, Slough, England, 1972).
9. Roca, R. & Roderio, A. *Arch. Zootec.* **20**, 235-247 (1971).
10. Bunch, T. D., Foote, W. C. & Spillet, J. J. *Theriogenology* **6**, 379-385 (1976).
11. Bratanov, C. & Dikov, V. *Proc. 4th int. Congr. Anim. Reprod.*, The Hague, 4, 744-749 (1962).
12. Hancock, J. L. *Proc. 5th int. Congr. Anim. Reprod.*, Trento, 3, 445-450 (1964).
13. Warwick, B. L. & Berry, R. O. *J. Hered.* **40**, 297-303 (1949).
14. Hancock, J. L., McGovern, P. T. & Stamp, J. T. *J. Reprod. Fert., Suppl.* **3**, 29-36 (1968).
15. Hancock, J. L. & McGovern, P. T. *Res. Vet. Sci.* **9**, 411 (1968).
16. Dent, J., McGovern, P. T. & Hancock, J. L. *Nature* **231**, 116-117 (1971).
17. Tucker, E. M., McGovern, P. T. & Hancock, J. L. *J. Reprod. Fert.* **27**, 417-425 (1971).
18. McGovern, P. T. *J. Reprod. Fert.* **34**, 215-220 (1973).
19. McGovern, P. T. *Br. vet. J.* **132**, 691-706 (1976).
20. Willadsen, S. M. *Nature* **277**, 298-300 (1979).
21. Gardner, R. L. *Nature* **220**, 596-597 (1968).
22. Tucker, E. M. & Clark, S. W. *Anim. Blood Grps biochem. Genet.* **11**, 163-183 (1980).

Experimental chimaeras—removal of reproductive barrier between sheep and goat

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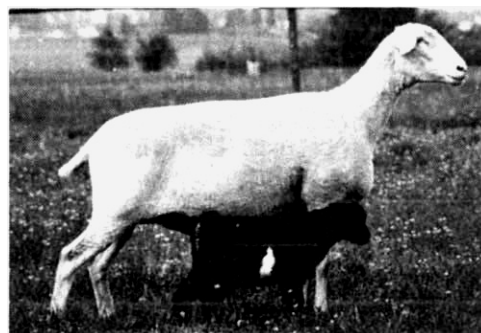


Fig. 1 Male goat-lamb by a Merino mother.

Following reciprocal embryo transfer between sheep and goats, the embryo of the foreign species is able to induce early pregnancy but the embryos do not survive beyond the first weeks of gestation^{1,2}. Similar results have been obtained from hybridization experiments on sheep and goats. While the causes for early embryonic death of hybrid eggs from donor sheep are unknown, the reciprocal event has been associated with immunological implications²⁻⁷. As a means of overcoming the reproductive barrier between sheep and goats, we have transferred interspecific chimaeric embryos. We report here the birth of a goat kid from a sheep mother.

Following oestrus synchronization and natural breeding, embryos were collected from super-ovulated donors on day 2 of pregnancy in sheep (onset of oestrus = day 0) and on day 3 of pregnancy in goats (second day of oestrus = day 0). The chronological difference between the embryos of the two species was about 24 h.

To produce interspecific chimaeric embryos we combined one sheep blastomere of the 4-cell stage with two goat blastomeres of the 8-cell stage, or two sheep blastomeres of the 'early' 8-cell stage with two goat blastomeres of the 'late' 8-cell stage in a common pig zona pellucida. To prevent wastage of cells the slit in the first zona pellucida was completely covered by a second one which surrounded the aggregated embryo with its first host zona. Micromanipulation was done as described previously⁸.

The embryos were then transferred to the oviducts of an intermediate recipient and were recovered again when blastula-

tion was expected. Embryos which reached the blastocyst stage were transferred to the uterine horns of the final sheep recipients. Diagnosis of pregnancy was by radioimmunological measurement of plasma progesterone in the recipients on day 18 and 50 of pregnancy.

The results of the experiments are summarized in Table 1. Of 15 interspecific chimaeric embryos analysed after recovery from the intermediate recipients, 9 had developed into a common blastocyst. Of four further embryos which reached the blastocyst stage, one blastomere of the aggregate did not continue to cleave, and in two cases two small blastocysts instead of one developed in their common zona pellucida.

Of 15 final recipients 8 did not return to oestrus and early pregnancy was confirmed by radioimmunoassay. The remaining seven animals returned to oestrus between day 21 and 36 but in all of the cases except one the plasma progesterone values were well above 2 ng ml⁻¹ on day 18. On day 50 plasma progesterone was above 2 ng ml⁻¹ in six animals.

Three recipients went to term and gave birth to two sheep- and one goat-lamb (Fig. 1). One of the sheep lambs was stillborn because of delayed parturition. The stillborn male sheep-lamb was derived from a combination of two sheep and two goat blastomeres, but in the common blastocyst one goat blastomere was not integrated. The female sheep-lamb was born after transfer of two blastocysts in one common zona pellucida. The goat-lamb developed after aggregation of one blastomere of a 4-cell sheep embryo and two blastomeres of an 8-cell goat embryo. All blastomeres had participated in the development of the common blastocyst.

Table 1 Development of interspecific chimaeric embryos

	Type of chimaeric embryo*	Development after transfer to intermediate recipients			Development after transfer to the final recipients			
		Common blastocyst	Blastocyst (one original blastomere did not cleave)	Two separate blastocysts	Plasma progesterone (ng ml ⁻¹)		Return to oestrus	Delivery
					day 18	day 50		
1	●●	+			7.6	3.2		
2	●●	+			7.2	3.9		
3	●●	+			6.6	5.0		♂ Goat-lamb
4	●●	+			3.4	3.7		
5	●●	+			0.1		+	
6	●●	+			3.3		+	
7	●●	+			6.9		+	
8	●●	+			8.2		+	
9	●●	+			3.1		+	
10	●●		+○		3.4		+	
11	●●		+●		4.2		+	
12	●●		+○		2.8	1.4		
13	●●		+○		2.2	1.5		♂ Sheep-lamb
14	●●			+	4.5	5.5		
15	●●			+	4.1	2.3		♀ Sheep-lamb

* ●, Sheep; ○, Goat.

Cytogenetic analysis, haemoglobin and transferrin typing, blood group serology, polyacrylamide gel electrophoretic analysis of blood and muscle proteins and breeding experiments gave no indications of chimaerism.

Thus, by micromanipulation it is possible to overcome the reproductive barrier between sheep and goats, but only when there is a protective mechanism which prevents recognition of the foreign fetus by the mother of the other species. It is assumed that at least the important parts of the placenta, particularly the chorion epithelium, which developed from the trophoblast of the chimaeric blastocyst, were derived from the sheep blastomere, and that the sheep trophoblast components were able to protect the goat embryonic cells from maternal immune rejection. This has been accomplished by combining chronologically different blastomeres. In the embryo which is formed, the younger component tends to give rise to the extra-embryonic membranes and the older component to the fetus. Recent

reports on developmental potential of sheep blastomeres⁹ and interspecific mouse chimaeras¹⁰ confirm these concepts. Equally, the experiments of S. M. Willadsen and C. B. Fehilly (personal communication) which resulted in the birth of sheep-goat chimaeras revealed the importance of constructing embryos so that the outside cells belong to the species into which the embryos have been transferred. Also, the endocrinological status indicates the sheep origin of the trophoblast. It is known that the goat placenta is not a source of progesterone as the sheep placenta is. After day 55 of pregnancy in sheep the survival of the fetus depends on proper progesterone production by the placenta¹¹.

The technique described here could be useful for rescuing endangered species.

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1. Warwick, B. L. & Berry, R. O. *J. Hered.* **40**, 297-303 (1949).
2. Hancock, J. L. & McGovern, P. T. *Res. vet. Sci.* **9**, 411-415 (1968).
3. Sokolovskaja, I. I. *Anim. Breeding. Abstr.* **10**, 204 (1940).
4. Alexander, G., Williams, D. & Bailey, L. *Aust. J. biol. Sci.* **20**, 1217-1220 (1967).
5. Hancock, J. L., McGovern, P. T. & Stamp, J. T. *J. Reprod. Fert. Suppl.* **3**, 29-36 (1968).
6. Dent, J., McGovern, P. T. & Hancock, J. L. *Nature* **231**, 116-117 (1971).

7. McGovern, P. T. *J. Reprod. Fert.* **34**, 215-220 (1973).
8. Meinecke-Tillmann, S. & Meinecke, B. *Zentbl. VetMed.* **A30**, 146-153 (1983).
9. Willadsen, S. M. & Fehilly, C. B. in *Fertilization of the Human Egg in Vitro* (eds Beier, H. M. & Lindner, H. R.) 353-357 (Springer, Berlin, 1983).
10. Rossant, J., Maurer, V. M. & Croy, B. A. *J. Embryol. exp. Morph.* **69**, 141-149 (1982).
11. Amoroso, E. C. & Perry, J. S. in *The Ovary* Vol. 2, 2nd edn (eds Lord Zuckerman & Weir, B. J.) 315-398 (Academic, New York, 1977).

An antigenic difference between cells forming early and late haematopoietic spleen colonies (CFU-S)

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Murine pluripotent haematopoietic stem cells have generally been assayed by their ability to form macroscopic colonies of haematopoietic cells in the spleens of heavily irradiated recipient mice (colony-forming unit-spleen or CFU-S assay)¹. However, recent evidence suggests that there are distinct subpopulations of CFU-S². Most spleen colonies present 7-8 days after injection consist of differentiated erythroid cells, contain no primitive myeloid or erythroid precursor cells and disappear from the spleen within 3 days, whereas the majority of colonies present at 10-12 days contain primitive precursors, are multilineal and cannot be detected at 7-8 days². Furthermore, many 10-day-old spleen colonies do not contain cells capable of forming spleen colonies in secondary irradiated recipients (an index of the self-renewal capacity of stem cells) whereas at day 14 almost all colonies contain these CFU-S³. These studies suggest that only when colonies are scored at or later than 11-12 days is the spleen colony technique adequate for the assay of multipotential stem cells. We now report an antigenic difference between subpopulations of CFU-S forming early (day 8) and late (day 14) spleen colonies which has been used to purify multipotential haematopoietic stem cells from murine bone marrow.

A cytotoxic IgG2b monoclonal antibody to a 'Qa-2 like' alloantigen, Qa-m2 (ref. 4), reacts with a minority (10-15%) of bone marrow cells⁴ (mostly lymphocytes⁵) and has been shown to kill, in the presence of complement, megakaryocyte colony-forming cells (MEG-CFC) but not granulocyte-macrophage (GM-CFC) or 7-day erythroid colony-forming cells (E-CFC)⁵. These experiments were extended to investigate whether more primitive haematopoietic cells (that is, CFU-S) express Qa-m2. Treatment with anti-Qa-m2 and complement had no effect on the capacity of C57BL/6 bone marrow cells

to form spleen colonies 8 days after injection into irradiated hosts (Table 1). However, spleen colony-forming ability was progressively inhibited at later time points. By day 14, spleen colony numbers were reduced by 82% compared with the number formed by cells treated with a negative control antibody, anti-Ly-2.1 (ref. 6), and complement. In contrast, treatment with monoclonal anti-H-2.2 and complement (positive control) abolished colony formation at all times. These results suggest progressive loss of Qa-m2 expression with differentiation from more primitive multipotential cells (day 14 CFU-S) to those with restricted potential (day 8 CFU-S).

Since the CFU-S assay may not adequately predict the capacity of haematopoietic tissue to repopulate the bone marrow of lethally irradiated recipients⁷, the effect of anti-Qa-m2+C on this property was studied (Table 2). Anti-Qa-m2+C

Table 1 Effect of anti-Qa-m2 and complement (C) on spleen colony formation at various times after injection of bone marrow cells

Days after injection of cells	CFU-S/ 1×10^5 marrow cells*			% Reduction by anti-Qa-m2 + C (no. of experiments)†
	Anti-H-2.2 + C	Anti-Ly 2.1 + C	Anti-Qa-m2 + C	
8	0	22.4 ± 3.0	21.1 ± 2.9	7 (5)
10	0	19.6 ± 2.0	15.5 ± 2.3	20 (3)
11	0.2 ± 0.4	19.3 ± 1.2	10.8 ± 1.0	44 (2)
12	0.3 ± 0.5	15.3 ± 2.1	4.2 ± 2.6	71 (3)
14	0	18.6 ± 2.8	3.1 ± 2.0	82 (4)

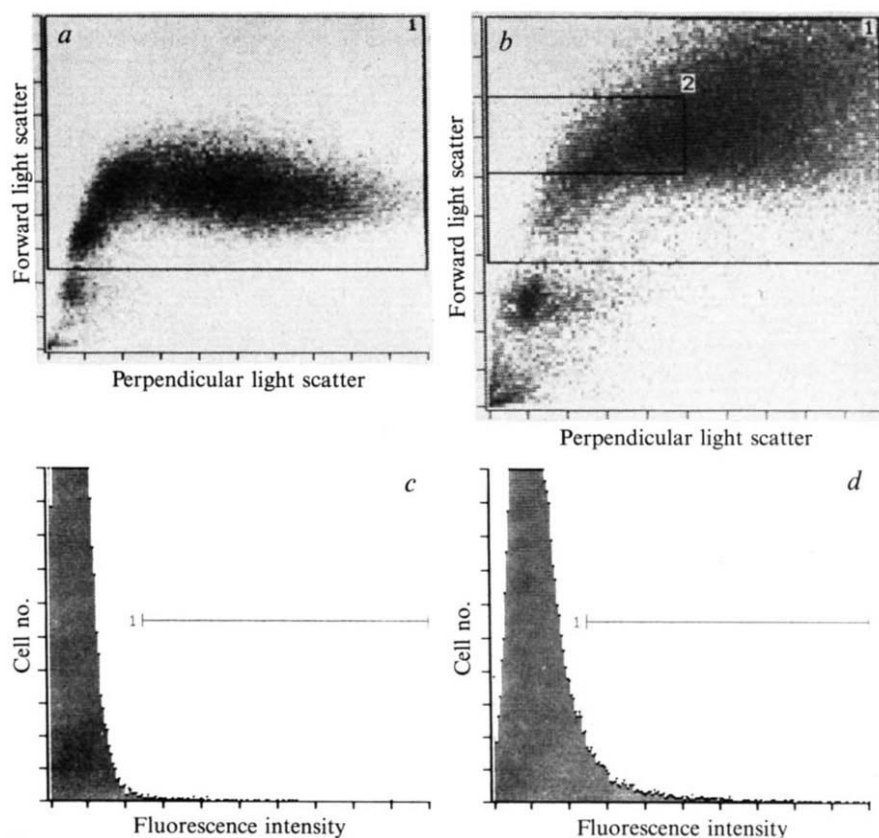
C57BL/6 bone marrow cells (2×10^6) in 0.1 ml HEPES-buffered alpha medium with 10% heat-inactivated fetal calf serum (FCS) were incubated with monoclonal anti-Qa-m2 (serum from hybridoma-bearing mice, final dilution 1:100), monoclonal anti-H-2.2 serum (1:100 dilution, positive control) or monoclonal anti-Ly-2.1 serum⁶ (1:100 dilution, negative control) for 30 min on ice. The concentration of anti-Qa-m2 serum used was > 1,000-fold greater than that required for maximal lysis of MEG-CFC⁵. The cells were washed, resuspended in 0.4 ml absorbed rabbit serum (diluted 1:8) as a source of complement (C), and incubated at 37 °C for 45 min. The cells were washed in phosphate-buffered saline (PBS) and injected into groups of 7-10 syngeneic irradiated (825R) specified-pathogen free recipients. From 3×10^4 (for day-14 colonies) to 1×10^5 cells (day-8 colonies) were given to each mouse. At the indicated times after injection, mice were killed by cervical dislocation. Their spleens were removed, fixed in Bouin's fixative, and surface macroscopic colonies were counted.

* Results from a typical experiment (mean ± 1 s.d.).

† Comparison is with anti-Ly-2.1 + C-treated cells (negative control). Mean per cent reduction from indicated number of experiments (in parentheses) is shown. The spleens of uninjected irradiated control mice contained < 1 colony per 10 spleens at each time point. Treatment of C57BL/6 bone marrow cells as described above produced $9.8 \pm 3.2\%$ (mean ± s.d.) lysis of anti-Qa-m2 + C treated cells compared with $7.3 \pm 2.1\%$ lysis after treatment with anti-Ly-2.1 + C (three experiments)⁵.

Fig. 1 *a*, Cytogram of lymphocyte-depleted normal marrow cells according to forward and 90° light scatter characteristics. *b*, Cytogram of 5FU8BM cells similarly analysed. *c*, *d*, Examples of fluorescence-intensity profiles for cells in region 2 of 5FU8BM: *c*, treated with anti-Ly-2.1 (negative control) and *d*, treated with anti-Qa-m2. The horizontal bar indicates the region in which cells were sorted as Qa-m2⁺.

Methods: Normal C57BL/6 bone marrow cells (NBM) or marrow cells from mice treated 8 days previously with 5-fluorouracil (150 mg kg⁻¹ intravenously) and endotoxin (*Escherichia coli* 0111:B4 (Difco), 6 µg per mouse intraperitoneally) (5FU8BM) were depleted of lymphocytes, red cells and dead cells as described⁵. Briefly, marrow cells were treated with monoclonal anti-Thy-1.2 serum (1:100 dilution), washed, rosetted with sheep red blood cells coupled with sheep anti-mouse Ig, and depleted of rosetted lymphocytes (T and B), red cells, dead cells and some neutrophils by centrifugation through an Isopaque-Ficoll gradient (ρ 1.090 g cm⁻³)⁵. The lymphocyte-depleted marrow interface cells were treated with anti-Qa-m2 serum (1:100 dilution) for 30 min on ice, washed three times and developed for indirect immunofluorescence by incubating with fluoresceinated F(ab')₂ goat anti-mouse IgG (Cappel Laboratories) (1:20 dilution) for 30 min on ice. Cells were washed and suspended in phosphate-buffered saline containing 1% bovine serum albumin and 0.02% sodium azide before analysis and sorting on an Ortho Cytofluorograph System 50L cell sorter. Unsorted and sorted fractions were assayed for their content of day-8 and day-14 CFU-S in lethally irradiated syngeneic recipients (Table 1). Cells were sorted on the basis of fluorescence only in large rectangular windows (region 1) in *a* and *b* (that is, gating out only dead cells which fluoresce non-specifically) or by three-parameter sorting by selecting fluorescent cells in a region (region 2 in *b*) enriched for CFU-S in 5FU8BM by light scatter (forward and 90°) characteristics, based on preliminary experiments (unpublished). Background fluorescence (that is <0.5% of cells) in each region was set using an aliquot of lymphocyte-depleted marrow cells (NBM and 5FU8BM) treated with anti-Ly-2.1 serum (negative control, 1:100 dilution) but otherwise treated as above. Anti-Qa-m2-treated cells with fluorescence intensity greater than background in each region were sorted as Qa-m2⁺ and the remainder of cells in each region sorted as Qa-m2⁻.



treatment significantly reduced the ability of bone marrow cells to reconstitute haematopoiesis, as measured by platelet counts, marrow cellularity and the femoral content of MEG-CFC and GM-CFC in recipients 14 days after injection of cells. Since anti-Qa-m2 with complement has no significant direct cytotoxic effect on GM-CFC or E-CFC (ref. 5), the reduction in marrow reconstituting ability probably reflects complement-mediated lysis of more primitive multipotential cells (such as day 14 CFU-S or 'pre-CFU-S') which generate these committed progenitor cells. The smaller reduction in repopulation indices (~50%) compared with reduction of day 14 CFU-S (~80%, Table 1) produced by anti-Qa-m2 and complement probably reflects incomplete regeneration from residual undamaged primitive precursors over the 14-day assay period.

As most C57BL/6 bone marrow cells are Qa-m2⁻ (refs 4, 5) the anti-Qa-m2 antibody was used to separate subpopulations of CFU-S and to purify day 14 CFU-S from bone marrow by indirect immunofluorescence using a cell sorter (Fig. 1, Table 3). All T-lymphocytes and ~25% of B-lymphocytes in C57BL/6 mice are Qa-m2⁺ (ref. 4). Therefore normal bone marrow was first depleted of lymphocytes (plus erythrocytes, dead cells and some neutrophils) using a rosetting procedure (ref. 5 and Fig. 1). The lymphocyte-depleted bone marrow cells contained >80% of day 8 and day 14 CFU-S (Table 3). The cells in this fraction were treated with anti-Qa-m2 and stained by indirect immunofluorescence. Viable cells were sorted into Qa-m2⁺ and Qa-m2⁻ fractions using lymphocyte-depleted marrow cells treated with a negative control antibody to set background fluorescence (Fig. 1). The Qa-m2⁺ fraction (~3% of unfractionated marrow cells) contained 65% of sorted day 14 CFU-S (representing 15-fold enrichment compared with unfractionated marrow) but only 6% of sorted day 8 CFU-S (1.6-fold enriched) most of which were Qa-m2⁻ (Table 3).

To further purify day 14 CFU-S, we used bone marrow obtained from mice treated 8 days previously with 5-fluorouracil (a cycle-active drug which preferentially kills proliferating cells) and endotoxin (which enhances marrow recovery⁸) (Fig. 1). This regenerating marrow (5FU8BM) contains supranormal numbers of CFU-S (ref. 8) and is enriched for these cells (Table 3). As found with normal marrow, >80% of day-8 and day-14 CFU-S were recovered in the lymphocyte-depleted 5FU8BM fraction. Single parameter (Qa-m2⁺ fluorescence) sorting of viable lymphocyte-depleted cells resulted in 15-fold enrichment for day-14 CFU-S. However the fluorescent Qa-m2⁺ fraction contained only 39% of sorted day-14 CFU-S (and <1% of sorted day-8 CFU-S) (Table 3). The reason for the smaller proportion of day-14 CFU-S in the Qa-m2⁺ cell fraction from regenerating marrow compared with normal marrow is not known. It is possible that Qa-m2 expression by these cells may be related to their cell-cycle status. We are currently investigating this possibility. Based on prior studies of the light-scattering characteristics of CFU-S in 5FU8BM (unpublished observations), fluorescent (Qa-m2⁺) cells were selected in a region gated according to forward and perpendicular light scatter (Region 2, Fig. 1b). This fraction was further enriched for day-14 CFU-S (to ~30-fold) but not for day-8 CFU-S, and contained 73% of the Qa-m2⁺ day-14 CFU-S (24% of initial) (Table 3). The final recovery of day 14 CFU-S in this fraction was ~15–20% of the initial day 14 CFU-S in unfractionated 5FU8BM after all fractionation procedures (after taking into account losses of cells during sorting, washing and so on).

The 24-h spleen-seeding efficiencies (*f*) of day-8 and day-14 CFU-S in 5FU8BM, determined as described^{3,9,10}, were 0.03 and 0.035, respectively. The corresponding values for 2-h *f* were 0.09 and 0.106. If, as seems likely¹¹, *f* is the same for enriched populations of CFU-S as for those in unfractionated marrow

Table 2 Effect of anti-Qa-m2 and complement (C) on capacity of bone marrow cells to reconstitute haematopoiesis in lethally-irradiated mice

Treatment of cells	Day-14 post-825 rad			
	Platelet count ($\times 10^9/1$)	Viable nucleated cells per femur ($\times 10^{-6}$)	GM-CFC per femur ($\times 10^{-3}$)	MEG-CFC per femur ($\times 10^{-2}$)
Anti-Ly-2.1+C (1×10^6 cells per mouse)	335 $\pm$ 27 (10)	14.1 $\pm$ 2.5 (10)	8.7 $\pm$ 2.1 (10)	6.0 $\pm$ 1.2 (10)
Anti-Qa-m2+C (1×10^6 cells per mouse)	162 $\pm$ 15 (10)	7.3 $\pm$ 1.3 (10)	3.8 $\pm$ 1.0 (10)	3.1 $\pm$ 0.8 (10)
Radiation control (no cells)	40 $\pm$ 3 (5)	1.2 $\pm$ 0.3 (5)	0 (5)	0 (5)

C57BL/6 bone marrow cells were treated with anti-Qa-m2 + C or anti-Ly-2.1 (negative control antibody) + C as described (Table 1) and injected into irradiated (825R) syngeneic hosts (1×10^6 cells per mouse). Fourteen days later, mice injected with cells and radiation controls receiving no cells were bled from the retroorbital plexus, then killed by cervical dislocation. Cells were obtained from the femurs of individual mice, counted, and assayed for megakaryocyte colony-forming cells (MEG-CFC) and granulocyte-macrophage colony-forming cells (GM-CFC) as described⁵. Femoral CFC content was calculated from the mean of four replicate culture plates per individual mouse cell suspension. Platelets were counted by phase microscopy. Results (mean $\pm$ 1 s.e.m.) of a single experiment are shown. The numbers in parentheses indicate number of mice. Similar results were obtained in two additional experiments.

Table 3 Separation of CFU-S populations and enrichment for day-14 CFU-S using monoclonal anti-Qa-m2 and cell sorting

Cell fraction	Normal bone marrow			5FU8 bone marrow		
	Initial viable cells %	CFU-S per 1×10^5 cells		Initial viable cells %	CFU-S per 1×10^5 cells	
		Day 8	Day 14		Day 8	Day 14
Unfractionated unsorted marrow	100	17.4	18.5	100	97	110
Lymphocyte-depleted marrow	57	27.2	27.9	45	179	206
Qa-m2 ⁻ viable cells (region 1, Fig. 1a and b)	53.7	25.9	9.8	42.8	172	111
Qa-m2 ⁺ viable cells (region 1, Fig. 1a and b)	3.3	28.5	285	2.2	32	1,660
Qa-m2 ⁺ cells selected by scatter characteristics for CFU-S (region 2, Fig. 1b)	ND	ND	ND	0.8	75	3,250

Bone marrow cells (NBM or 5FU8BM) were depleted of lymphocytes, treated with anti-Qa-m2, developed for indirect immunofluorescence, and sorted on the basis of fluorescence only (region 1) or by three parameter sorting (that is, forward and perpendicular light scatter characteristics and fluorescence (region 2)) as described (Fig. 1). The relative proportions of CFU-S in the various fractions (per cent of initial unfractionated unsorted marrow) were determined by multiplying the number of CFU-S per 1×10^5 cells by the proportion of viable cells in that fraction and dividing by the frequency of CFU-S in unsorted unfractionated marrow (for results see text). Results are typical of 3–6 similar experiments. ND, not determined.

and the 2-h f values are used, then at least 30% of cells in the most highly enriched fraction of 5FU8BM are day-14 CFU-S and ~0.8% are day-8 CFU-S. However, if the more generally accepted^{9,10} 24-h values for f are used, ~90% of these cells are day-14 CFU-S (and ~2% are day-8 CFU-S). More than 98% of the cells in this fraction were undifferentiated mononuclear cells with moderately high nucleus-cytoplasm ratio, immature chromatin pattern, several nucleoli and agranular basophilic cytoplasm. Culture of these cells in agar⁵ showed that ~5% were MEG-CFC, ~1–2% were GM-CFC and 2–3% were mixed E-CFC. Thus, highly purified multipotential stem cells (day-14 CFU-S), separated from the bulk of day-8 CFU-S, committed progenitor cells and other marrow cells, were obtained with reasonable yield from 5FU8BM.

Our results clearly demonstrate an antigenic difference between functionally distinct subpopulations of CFU-S, and strongly suggest that Qa-m2 is, or is part of, a differentiation antigen for multipotential haematopoietic stem cells. An intriguing but as yet untested possibility is that the Qa-m2 determinant may play a functional role in haematopoietic differentiation. Previous studies⁵ and studies in progress (unpublished observations) indicate that differences in Qa-m2 expression can be used to separate MEG-GFC and mixed erythroid-CFC (Qa-m2⁺) from GM-CFC and 7-day E-CFC (Qa-m2⁻) suggesting that the former two types of CFC are more closely related to the multipotential stem cell than the latter

committed progenitor cells. Thus the anti-Qa-m2 antibody should be a valuable tool for investigating the earliest steps in haematopoietic differentiation, including the order of restriction of potentialities in stem cell progeny¹². Highly purified multipotential stem cells, obtained as described here, will be useful in investigation of the process of commitment to a line of differentiation, and in exploring the relationship between CFU-S and other primitive haematopoietic cells^{7,13,14}. However, as day-14 CFU-S in 5FU8BM may have properties different from those in unstimulated normal bone marrow, we are currently attempting to further purify these cells from normal marrow using density gradients¹⁵ and depletion of mature marrow cells with monoclonal antibodies¹⁶ before cell sorting.

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1. Till, J. E. & McCulloch, E. A. *Radiat. Res.* **14**, 213–222 (1961).
2. Magli, M. C., Iscove, N. N. & Odartchenko, N. *Nature* **295**, 527–529 (1982).
3. Siminovich, L., McCulloch, E. A. & Till, J. E. *J. cell comp. Physiol.* **62**, 327–336 (1963).
4. Hogarth, P. M., Crewther, P. E. & McKenzie, I. F. C. *Eur. J. Immun.* **12**, 374–379 (1982).
5. Harris, R. A., Hogarth, P. M., McKenzie, I. F. C. & Penington, D. G. *Expl. Hemat.* **11**, 527–541 (1983).
6. Hogarth, P. M., Edwards, J., McKenzie, I. F. C., Goding, J. W. & Liew, F. Y. *Immunology* **46**, 135–144 (1982).
7. Hodgson, G. S. & Bradley, T. R. *Nature* **281**, 381–382 (1979).
8. Hodgson, G. S. & Bradley, T. R. *Cancer Treat. Rep.* **63**, 1761–1769 (1979).

9. Lahiri, S. K., Keizer, H. J. & van Putten, L. M. *Cell Tissue Kinet.* **3**, 355–362 (1970).
10. Monette, F. C. & de Mello, J. B. *Cell Tissue Kinet.* **12**, 161–175 (1979).
11. Visser, J. W. M. & Eliason, J. F. *Cell Tissue Kinet.* **16**, 385–392 (1983).
12. Nicola, N. A. & Johnson, G. R. *Blood* **60**, 1019–1029 (1982).
13. Nakahata, T. & Ogawa, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3843–3847 (1982).
14. Boggs, D. R., Boggs, S. S., Saxe, D. F., Gress, L. A. & Canfield, D. R. *J. clin. Invest.* **70**, 242–253 (1982).
15. Visser, J. W. M. & Bol, S. J. L. *Stem Cells* **1**, 240–249 (1981).
16. Hoang, T. et al. *Blood* **61**, 580–588 (1983).

Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurones

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The shape of a neurone—the projection and branching pattern of axons and dendrites—appears to be determined by a combination of intrinsic and environmental influences^{1–5}. We have previously shown that striatal target neurones influence the biochemical maturation of ascending mesencephalic dopamine (DA) cells in culture^{6,7}, as well as the elongation rate of DA neurites⁸. Using a similar approach in which the morphology of individual DA cells can be studied after ³H-DA uptake and autoradiography, we now report on *in vitro* neurone-glia interactions and show that glial cells exert a morphogenetic effect on DA neurones. Dopaminergic neurones from the mesencephalon were plated on glial monolayers prepared either from the striatal or the mesencephalic region of the embryonic brain. On mesencephalic glial cells the majority of DA neurones develop a great number of highly branched and varicose neurites, whereas on striatal glia they only exhibit one long, thin and rather linear neurite. These results demonstrate that glial cells from two different brain regions have distinct properties which could be used to define neuronal polarity observed *in vivo*.

Cultures of glial cells from mouse embryos were generated from the mesencephalon (ED 13) and the striatum (ED 15) and grown for 3 weeks as described in detail in Fig. 1 legend. In

such cultures, most neurones have died and the non-neuronal cells form a confluent monolayer (Fig. 3). Indeed autoradiography after ³H-DA uptake or immunocytochemistry after incubation with an antibody against the 70 K subunit of the neurofilament triplet did not reveal the presence of surviving neurones. Mesencephalic cells (ED 13) containing a subpopulation of DA neurones were plated on these preformed monolayers and their morphology was examined after 1, 2, 3 and 10 days in culture following ³H-DA uptake and autoradiography. Already after 1–2 days in culture DA neurones grown on glial cells from these two brain areas exhibit strikingly different morphologies. On a glial monolayer generated from the striatum, most cells are either ovoid or multipolar as described *in vivo* and *in vitro*^{9–11}, they usually extend one long linear process at one pole and one or a few shorter ones at the opposite pole or around the cell soma. This type of morphology is illustrated in Fig. 1a in which four cells have been photographed. However, when grown on a glial monolayer originating from the mesencephalon they have a great number of highly branched smooth or varicose processes (Fig. 1b). Figure 2 shows the differences in the morphology of DA neurones observed in both culture conditions. The number of neurites on glial mesencephalic monolayers as well as their thick varicose aspect has led us to call them 'highly branched' processes. In contrast, the thin and almost unique neurite that elongates on striatal glial cells will be called 'poorly branched'. Similar results were obtained when DA neurones were identified by immunocytochemistry using an antibody against tyrosine hydroxylase.

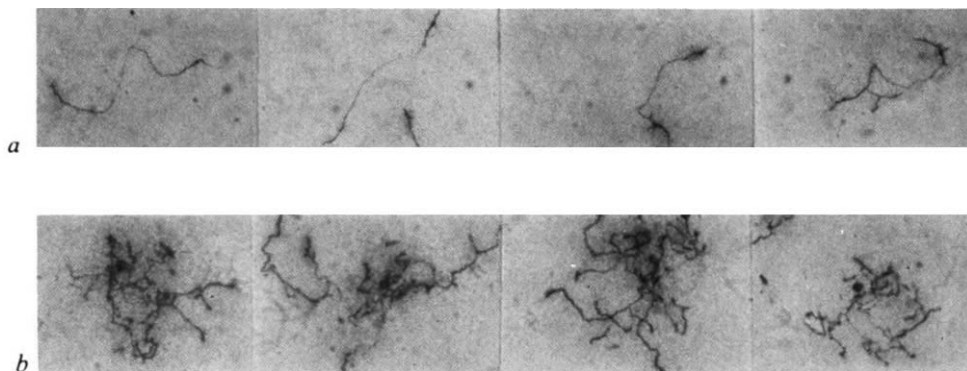
As already pointed out this dramatic difference between the morphologies of DA cells is clearly visible as early as one day after the beginning of the culture. Table 1 shows the number of neurones displaying one or the other configuration after various times in culture. Since in both culture conditions a small constant number (15%) of DA cells does not extend any neurite, we have only included neurites displaying cells in this table. The data show that the number of labelled DA neurones is identical after 2 days in culture on both monolayers. It slightly increases after 3 days probably because some neurones had not yet acquired the capacity to take up the neurotransmitter after 2 days *in vitro*. After 10 days, the number of surviving neurones is lower on both glial monolayers. The percentage of DA neurones presenting a 'highly branched' arborization remains constant on mesencephalic glial monolayers (60, 74 and 54% at 2, 3 and 10 days respectively). This is not the case on striatal glial monolayers, since, although 100% of the neurones develop one long neurite after 2 and 3 days *in vitro*, after 10 days this percentage falls to 34%, and 66% exhibit 'highly branched'

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Fig. 1 a, Visualization of four dopaminergic neurones after 2 days *in vitro* on a monolayer of striatal glial cells (×46). b, Visualization of four dopaminergic neurones after 2 days *in vitro* on a monolayer of mesencephalic glial cells (×46).

Methods: Cells from the mesencephalon and the striatum of 13- and 15-day-old Swiss mouse embryos were prepared as already described^{6–7}, and plated at the concentration of 10⁶ cells per dish in Falcon dishes coated with polyornithine (Sigma, 40,000 MW, 1.5 µg ml⁻¹).

Glial monolayers were obtained by culturing the cells for 3 weeks in nutrient mixture F12, minimum essential medium (Gibco, 1:1) complemented with 10% inactivated fetal calf serum (Serumaid), 3.3 × 10⁻² M glucose, 2 × 10⁻³ M glutamine and 0.3 × 10⁻² M sodium bicarbonate. The culture medium was not changed for 1 week and then changed 3 times a week. Freshly dissociated rostral mesencephalic 13-day-old embryonic cells (5 × 10³ to 10⁶) were seeded on these monolayers and the culture was pursued for 2 more days. Dopaminergic neurones were examined by autoradiography. Cells were incubated at 37 °C for 1 h with 5 × 10⁻⁷ M ³H-DA (6 Ci mmol⁻¹, NEN) in 1-ml phosphate buffer supplemented with 10⁻³ M magnesium, 10⁻³ M calcium and 3.3 × 10⁻² M glucose (PBS). After three washes with cold PBS, the cells were fixed for 1 h at room temperature with 1.5% glutaraldehyde (Taab Laboratories) in PBS, washed four times with PBS, air dried and covered with Kodak NTB2 emulsion. Autoradiograms were developed 2 weeks later using Kodak Dektol. No labelling was seen when the cells were incubated in the presence of 5 × 10⁻⁶ M benztropine (Merck), a potent inhibitor of ³H-DA uptake into dopaminergic neurones.



processes. The *in vitro* development of mesencephalic glial cells seeded together with the neurones could provide an explanation for this observation.

Since glial monolayers are important in neuronal morphogenesis, we analysed their cellular composition by immunocytochemistry using an antibody against the glial fibrillary acidic protein (GFAP), a specific marker for astrocytes. The morphology of mesencephalic and striatal non-neuronal cultures is not very different (Figs 3a and b): the cells are not equally stained with the antibody, a fact that demonstrates some heterogeneity in the level of expression of the glial filament. The weak fluorescence of some cells cannot be attributed to cross-reactions with vimentin. This was checked by the absence

of GFAP labelling of several virally transformed cell lines known to synthesize high amounts of vimentin¹² as well as by immunoblotting experiments (not shown). We have shown that 8-day old mesencephalic primary cultures contain a large proportion of vimentin-positive cells which cannot be labelled with GFAP antisera in double-labelling experiments nor with anti-fibronectin or anti-galacto cerebrosides antibodies¹³. Although we do not exclude the presence of other cell types such as immature oligodendrocytes, microglia or epithelial cells we agree with several authors^{14,15} that the non-neuronal monolayers are mainly composed of astroblasts at various maturation stages and astrocytes.

Glial cells, peripheral and central, play important roles in promoting neuronal survival^{16,17}, neurite outgrowth^{18,19}, differentiation^{20,21} and neurotransmitter choice^{22,23}. Our study demonstrates that glial cells from two different embryonic brain areas have distinct and specific morphogenetic effects on the polarity and arborization of mesencephalic ascending DA neurones.

The morphological differences between DA cells plated on mesencephalic or striatal glial substratum could reflect different

Table 1 Morphology of dopaminergic neurones

Days <i>in vitro</i>	Arborization of dopaminergic neurones in:			
	Mesencephalic glial monolayer		Striatal glial monolayer	
	Poorly branched	Highly branched	Poorly branched	Highly branched
2	458 (40%)	705 (60%)	1,103 (100%)	—
3	340 (36%)	1,008 (74%)	1,790 (100%)	—
10	210 (46%)	251 (54%)	327 (34%)	548 (66%)

Number of dopaminergic neurones exhibiting different morphologies when grown on the two different types of glial cell monolayers. This experiment has been repeated three times and about the same percentages of the two morphologies have been found in all experiments at two concentrations of freshly dissociated mesencephalic cells (5×10^5 and 10^6).

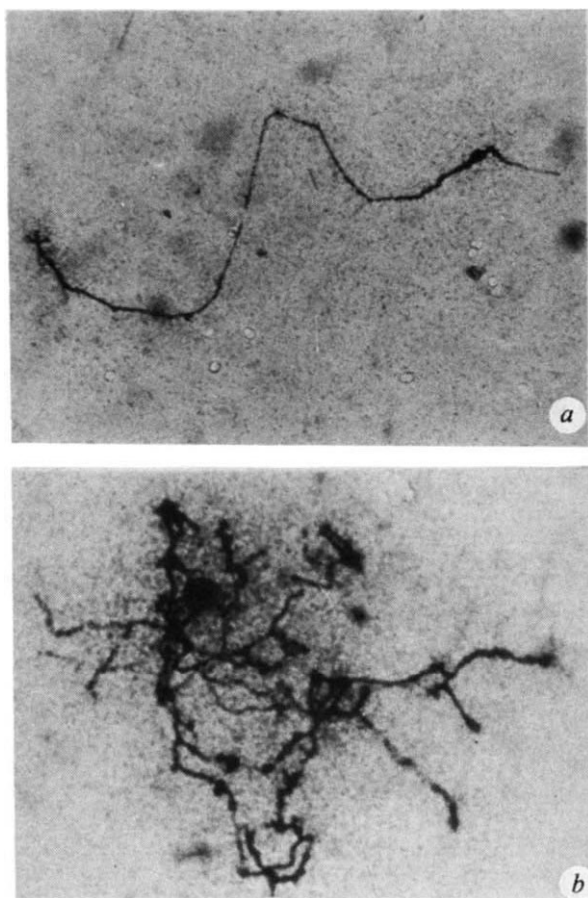


Fig. 2 Higher magnification of two neurones presented in Fig. 1. *a*, Dopaminergic neurones after 2 days *in vitro* on a monolayer of striatal glial cells ($\times 300$). *b*, Dopaminergic neurone after 2 days *in vitro* on a monolayer of mesencephalic glial cells ($\times 300$).

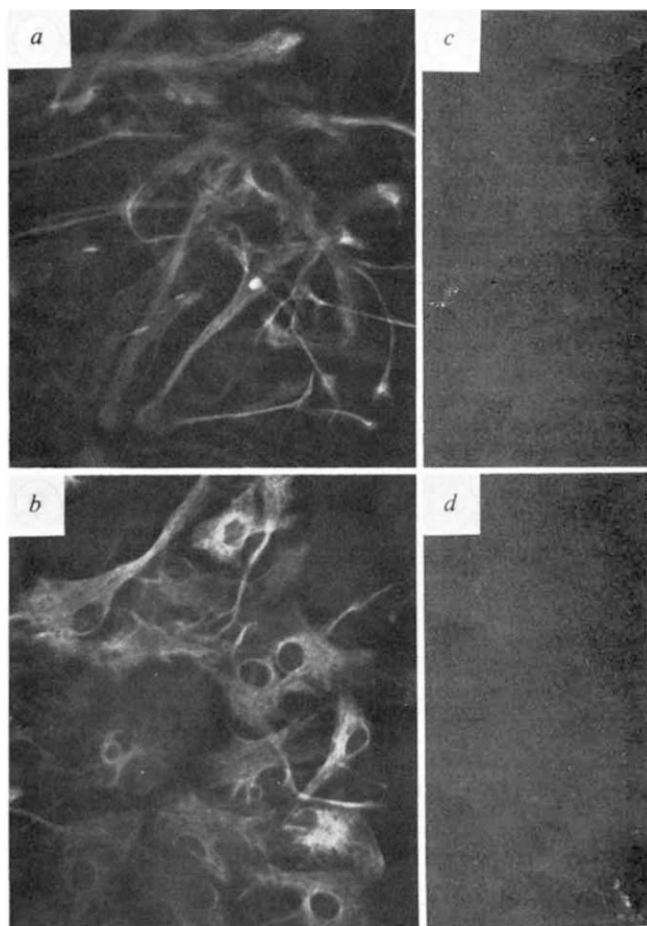


Fig. 3 Mesencephalic (*a*, *c*) and striatal (*b*, *d*) non-neuronal monolayers were prepared as described in Fig. 1. After 3 weeks in culture they were rinsed with PBS and permeabilized with a mixture of 3.7% formaldehyde and 1% methanol (Merck) for 1 h at room temperature. After several washes with PBS the cells were incubated 1 h at 37 °C with (*a*, *b*) or without (*c*, *d*) an antibody against glial fibrillary acidic protein (GFAP), raised in the rabbit. The second incubation was performed at 37 °C for 30 min with a solution of fluorescein-conjugated goat anti-rabbit immunoglobulins (1:50, Nordic). All incubations were in PBS containing 10% fetal calf serum. The cultures were mounted in PBS, glycerol (1:1) before examination under a Leitz HM-Lux microscope using a HBO 50-W mercury lamp. Magnification ($\times 250$).

developmental stages achieved by the neurones. This would mean that mesencephalic glial cells synthesize molecules that stimulate DA neurone development. Alternatively, the different morphologies of DA cells on both glial monolayers could arise by the specific selection by these monolayers of subpopulations of DA neurones. This seems not to be the case: the same number of DA cells is observed after 2 days *in vitro* on both substrata and since neurones growing a 'poorly branched' neurite can slowly develop a 'highly branched' arborization when mesencephalic glial cells have multiplied on the striatal monolayer. A third more interesting explanation comes from the observation that the morphological pattern obtained *in vitro* strikingly coincides with the geometry of the mesostriatal DA neurones. Indeed, during development, DA nigral neurones send their axons rostrally toward the striatum whereas the dendritic arborizations develop caudally in the nigral pars reticulata. It could be that on striatal as well as on a subpopulation of mesencephalic glia (40%), the unique neurite would represent an axon while on another subpopulation of mesencephalic non-neuronal cells (60%) DA neurones would be able to extend dendritic branches, with or without axon. Hemmendinger *et al.*²⁴ have already suggested that the formation of 'axon-like' processes depends on the presence of striatal or cortical target

cells, whereas areas of extensive 'dendritic-like' arborizations are seen when DA neurones are in aggregates of mesencephalic cells only.

Since both neuronal morphologies can coexist in the same culture, either on mesencephalic glial cells or on striatal glial cells after 10 days when mesencephalic glia has been able to develop, diffusible molecules acting at long distances cannot be responsible for the observed phenomenon; close interactions between the neurite and its glial substratum are necessary.

In an earlier study we showed that striatal target cells are able to regulate *in vitro* the growth of DA neurones. In fact, DA processes transiently stop on these striatal target cells, while they ignore non-target mesencephalic or cerebellar cells⁸. It is, therefore, possible that direct contact is involved in stimulation and direction of axonal growth by striatal glial cells and recognition by axonal terminals of striatal target neurones. On the other hand the development of dendritic arborization could depend strictly on the signals provided by local mesencephalic glia.

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- Solomon, F. *Cell* **16**, 165-170 (1979).
- Bray, D. J. *Cell Biol.* **56**, 702-712 (1973).
- Benfey, M. & Aguayo, A. J. *Nature* **216**, 150-152 (1982).
- Berry, M., McConnell, P. & Sievers, J. *Curr. Topics devl Biol.* **15**, 67-101 (1980).
- Shankland, P., Bentley, D. & Goodman, C. S. *Devl Biol.* **92**, 507-520 (1982).
- Prochiantz, A., di Porzio, U., Kato, R., Berger, B. & Glowinski, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5387-5391 (1979).
- di Porzio, U., Daguet, M.-C., Glowinski, J. & Prochiantz, A. *Nature* **288**, 370-373 (1980).
- Denis-Donini, S., Glowinski, J. & Prochiantz, A. *J. Neuroscience* **3**, 2292-2299 (1983).
- Bjorklund, A. & Lindvall, O. *Brain Res.* **83**, 531-537 (1975).
- Fallon, J. H., Riley, J. N. & Moore, R. Y. *Neurosci. Letters* **7**, 157-162 (1978).
- Berger, B. *et al. Neuroscience* **7**, 193-205 (1982).
- Lazarides, E. *Nature* **283**, 249-256 (1980).
- Prochiantz, A., Delacourte, A., Daguet, M.-C. & Paulin, D. *Expl Cell Res.* **139**, 404-410 (1982).
- Sensenbrenner, M., Devilliers, G., Bock, E. & Porte, A. *Differentiation* **17**, 51-61 (1980).
- Lindsay, R. M., Barber, P. C., Sherwood, M. R. C., Zimmer, J. & Raisman, G. *Brain Res.* **243**, 329-343 (1982).
- Barde, Y. A., Edgar, D. & Thoenen, H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1199-1203, (1980).
- Lindsay, R. M. *Nature* **282**, 80-82 (1979).
- Adler, R. & Varon, S. *Devl Biol.* **86**, 69-80 (1981).
- David, S. & Aguayo, A. J. *Science* **214**, 931-933 (1981).
- Banker, G. A. *Science* **209**, 809-810 (1980).
- Touzet, N. & Sensenbrenner, M. *Devl Neurosci.* **1**, 159-163 (1978).
- Patterson, P. H. & Chun, L. L. Y. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3607-3610 (1974).
- Patterson, P. H. & Chun, L. L. Y. *Devl Biol.* **56**, 263-280 (1977).
- Hemmendinger, L. M., Garber, B. B., Hoffman, P. C. & Heller, A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1264-1268 (1981).

A dynorphinergic pathway of Leu-enkephalin production in rat substantia nigra

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The amino acid sequence of the opioid peptide Leu-enkephalin¹⁻³ is found within several larger peptides⁴⁻⁸, which are generated from the precursors proenkephalin and prodynorphin. Proenkephalin contains four copies of the sequence of Met-enkephalin, a single copy of the sequence of Leu-enkephalin and one copy each of two extended Met-enkephalin sequences⁹⁻¹¹. Proenkephalin contains three peptides— α -neo-endorphin, dynorphin A and dynorphin B¹²—the N-terminal sequences of which are identical to that of Leu-enkephalin. There is good evidence that the large amounts of Leu-enkephalin found in the adrenal medulla are generated from the precursor proenkephalin^{2,4,13,14}, but as yet prodynorphin has not been shown to be processed to yield Leu-enkephalin. We show here that the relatively high levels of Leu-enkephalin found in the rat substantia nigra are supplied by striatonigral axons and generated from the precursor prodynorphin.

During our studies of the regional distributions of prodynorphin-related peptides, we observed that the concentrations of α - and β -neo-endorphin were considerably higher than the concentrations of dynorphin A (plus dynorphin A(1-8)) or dynorphin B^{15,16}. This was especially striking in the substantia nigra and suggested to us that dynorphins A and/or B might be converted into smaller peptide species, including Leu-enkephalin. The fact that the level of Leu-enkephalin in the substantia nigra is more than twice as high as the level of Met-enkephalin-Arg-Gly-Leu, a peptide derived exclusively from proenkephalin (Table 1), also suggested that the Leu-enkephalin in this structure may not be derived exclusively from the proenkephalin precursor. Leu-enkephalin and Met-enkephalin-Arg-Gly-Leu are present in equimolar amounts in proenkephalin, and one may therefore expect their concentrations to be similar.

To determine the source of the Leu-enkephalin in the substantia nigra, we made unilateral knife cuts just rostral to the mesencephalon that transected descending fibres from the forebrain (Fig. 1, cut 2). These cuts resulted in a marked depletion of nigral α -neo-endorphin, dynorphin B and Leu-enkephalin, but did not affect levels of Met-enkephalin-Arg-Gly-Leu (Table 1). Thus, Met-enkephalin-Arg-Gly-Leu is probably not contained in descending inputs to substantia nigra. The dynorphin-related peptides and leu-enkephalin, on the other hand, must be in axons of neurones rostral to the substantia nigra.

In a second set of animals we made knife cuts that separate the globus pallidus from the caudate-putamen (Fig. 1, cut 1). These knife cuts also caused a substantial decrease in α -neo-endorphin, dynorphin B and Leu-enkephalin in the ipsilateral substantia nigra without altering the level of Met-enkephalin-Arg-Gly-Leu (Table 1). The same cuts resulted in decreases in

Table 1 Effects of various lesions on peptides in brain regions

Peptide	Treatment		Substantia nigra	Globus pallidus	Caudate
α -neo-endorphin	Rostral nigral Deaf.	Intact	6,496.8 $\pm$ 652.5 (8)	962.3 $\pm$ 167.3 (8)	352.6 $\pm$ 48.6 (8)
		Lesioned	942.6 $\pm$ 291.6* (8) -85%	951.1 $\pm$ 97.8 (8)	731.5 $\pm$ 113.9* (8) +107%
	GP Deaf.	Intact	7,195.5 $\pm$ 953.5 (8)	981.2 $\pm$ 91.9 (8)	379.1 $\pm$ 47.1 (8)
		Lesioned	1,053.3 $\pm$ 353.0* (8) -85%	324.9 $\pm$ 78.3* (8) -67%	786.3 $\pm$ 125.7† (8) +107%
Dynorphin-B	Rostral nigral Deaf.	Intact	2,019.8 $\pm$ 375.8 (8)	248.5 $\pm$ 54.0 (8)	173.8 $\pm$ 24.4 (5)
		Lesioned	300.4 $\pm$ 87.5* (8)	289.7 $\pm$ 65.4 (8)	339.8 $\pm$ 57.6‡ (5)
	GP Deaf.	Intact	2,133.7 $\pm$ 397.6 (8)		+95%
		Lesioned	291.9 $\pm$ 89.5* (8) -86%	Not determined	
Leu-enkephalin	Rostral nigral Deaf.	Intact	955.3 $\pm$ 99.8 (7)	3,525.6 $\pm$ 983.6 (7)	551.0 $\pm$ 70.2 (7)
		Lesioned	247.4 $\pm$ 62.7* (7) -74%	4,744.0 $\pm$ 998.0 (7)	628.9 $\pm$ 66.4 (7)
	GP Deaf.	Intact	784.6 $\pm$ 87.6 (7)	3,810.2 $\pm$ 746.4 (8)	420.4 $\pm$ 56.5 (8)
		Lesioned	211.1 $\pm$ 52.9* (8) -73%	471.5 $\pm$ 66.8* (8) -88%	793.9 $\pm$ 81.7† (8) +89%
Met-enkephalin-Arg-Gly-Leu	Rostral nigral Deaf.	Intact	415.3 $\pm$ 82.9 (7)	5,338.6 $\pm$ 874.6 (7)	1,450.3 $\pm$ 291.8 (8)
		Lesioned	341.7 $\pm$ 74.1 (8)	5,836.5 $\pm$ 1,006.8 (8)	1,830.7 $\pm$ 350.3 (8)
	GP Deaf.	Intact	334.9 $\pm$ 40.7 (8)	5,985.2 $\pm$ 755.4 (8)	1,001.1 $\pm$ 111.0 (8)
		Lesioned	385.1 $\pm$ 44.2 (8)	801.8 $\pm$ 144.3* (8) -87%	2,039.9 $\pm$ 210.6* (8) +104%

Brain areas investigated were substantia nigra, globus pallidus and caudate nucleus. Values are expressed as fmol peptide per mg protein. Data are means $\pm$ s.e.m. Lesioned side is compared with intact side by paired *t*-test: * $P < 0.001$, † $P < 0.01$, ‡ $P < 0.05$. When a percentage change is indicated the lesioned side and control sides differed significantly. Numbers in parentheses indicate the number of separate animals studied. GP, globus pallidus; Deaf., deafferentation.

Methods: Male Sprague-Dawley rats (200–250 g) were killed by decapitation (10 days after operation) and their brains removed. Tissue samples were microdissected from frozen 300- μ m sections of the brains²² after inspection of the lesions. Individual tissue samples were taken from the lesioned and control sides of the same section. Tissue samples were placed in Eppendorf tubes containing 200 μ l of 0.1 M HCl and homogenized by sonication. 20- μ l aliquots of the homogenates were taken for protein determination²³. The homogenates were centrifuged at 2,000g for 10 min at 4 °C, and the supernatants were transferred to 12 $\times$ 75 mm polystyrene tubes and evaporated to dryness in a vacuum centrifuge. The radioimmunoassay (RIA) procedure was that described in ref. 17 with some modifications. Samples were rehydrated in phosphate-buffered saline (pH 7.6). Antisera were used at final dilutions of 1:60,000 for α -neo-endorphin, and Leu-enkephalin, 1:100,000 for dynorphin B and 1:75,000 for Met-enkephalin-Arg-Gly-Leu, which resulted in 30–45% noncompetitive binding of the trace. Each sample was incubated in a 500- μ l volume containing 300 μ l of sample in assay buffer, 100 μ l of ¹²⁵I-labelled opioid peptide (~6,000 c.p.m.). The tubes were then incubated at 4 °C for 16–24 h. In RIAs for α -neo-endorphin and dynorphin B, separation of bound from free peptide was effected by addition of 1 ml of dextran-coated charcoal. A double-antibody RIA for Leu-enkephalin and Met-enkephalin-Arg-Gly-Leu was performed as described here. Normal rabbit serum (NRS) was also added to the first antibody solution to give a total NRS concentration of 1%. 100 μ l of this solution were added to all except 'nonspecific binding' tubes which received 100 μ l of 1% NRS in assay buffer. Reagents were mixed and the tubes incubated for 16–24 h. 100 μ l of goat anti-rabbit γ -globulin diluted with assay buffer to a concentration sufficient for maximal precipitation were added to all tubes. Reagent was mixed and the tubes were incubated for 16–24 h. After incubation with the second antibody, all tubes were centrifuged for 20 min at 2,000g at 4 °C. The supernatant was aspirated and pellets were counted in a γ -counter. The RIA sensitivities were less than 4 pg per tube. The specificities of the antisera for dynorphin B²⁴, α -neo-endorphin²⁵, Leu-enkephalin²⁵ and Met-enkephalin-Arg-Gly-Leu²⁶ have been described elsewhere. The α -neo-endorphin antiserum does not recognize the enkephalins and dynorphins at 1 μ M concentration. The dynorphin B antiserum does not cross-react with the enkephalins, dynorphin A, dynorphin A(1–8) or neo-endorphins at 1 μ M concentration. The Leu-enkephalin antiserum cross-reacts with Met-enkephalin to 0.35% and shows <0.01% cross-reactivity with dynorphins or neo-endorphins at 1 μ M concentration. The Met-enkephalin-Arg-Gly-Leu antiserum cross-reacts <0.005% with Met-enkephalin, Met-enkephalin-Arg, Met-enkephalin-Arg-Arg, Met-enkephalin-Arg-Phe, BAM 12P, dynorphin A(1–8), α -neo-endorphin and human β -endorphin at 1 μ M concentration.

α -neo-endorphin, Leu-enkephalin and Met-enkephalin-Arg-Gly-Leu in the globus pallidus (Table 1). This finding confirms an earlier observation of the (pro)enkephalinergic striatopallidal pathway¹⁷ and suggests that there are also dynorphinergic striatonigral and striato (or cortico)-pallidal pathways.

Dynorphin-containing cell bodies have been reported in the caudate¹⁸. Indeed, Vincent *et al.* and Palkovits *et al.* have described a striatonigral dynorphin system^{19,20}. We sought to visualize such neurones after globus pallidus isolation (cut 2),

reasoning that a dynorphin build-up in axons and perikarya (see Table 1) might facilitate immunocytochemical staining. In these brains we demonstrate dynorphin B immunoreactivity building up in axons on the proximal (caudate) side of the cut (Fig. 2a) and a dense network of stained nerve fibres and numerous cell bodies in the head of the caudate nucleus at some distance from the lesion (Fig. 2b–d). No cell bodies and very few immunoreactive fibres were observed on the intact side of the brain. There was a significant decrease in immunostaining in the substantia nigra on the side of the lesion (data not shown).

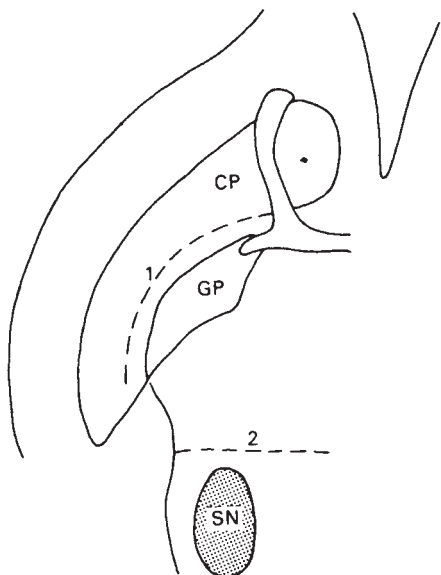


Fig. 1 Schematic representation of a horizontal section of the rat brain. CP, caudoputamen; GP, globus pallidus; SN, substantia nigra. 1, Unilateral knife cut isolating the caudate-putamen from globus pallidus. A Halasz knife²⁷ with a 2-mm radius and 3-mm height (the distance from the sleeve to the knife tip) was used to make this cut. The animal's head was fixed in a Kopf stereotaxic device (5°, nose down), and a rectangular flap of bone was removed from the skull. With the knife facing forward, the knife tip was placed 0.5 mm rostral to the bregma (~8.5 mm rostral to the interauricular line) and 2.5 mm lateral to the midline. The tip of the knife was lowered 7 mm beneath the surface of the dura and rotated 90° laterally. The knife was then moved caudally 2.5 mm, rostrally 2.5 mm and rotated medially 90° so that it was in its starting position in the brain. Finally, the knife was moved medially 2 mm, laterally 2 mm and withdrawn from the brain. 2, Unilateral mesencephalic cut transecting descending axons from caudate-putamen. This lesion was made with a 3-mm knife. The animal's head was fixed in a Kopf stereotaxic device (5°, nose down). The medial tip of the knife was placed 1 mm lateral to the midline 4.2 mm rostral to the interauricular line, and inserted ventrally until it touched the base of the brain. The animals were allowed to recover for 10 days, then they were decapitated and their brains were removed. Placement of all lesions was verified histologically.

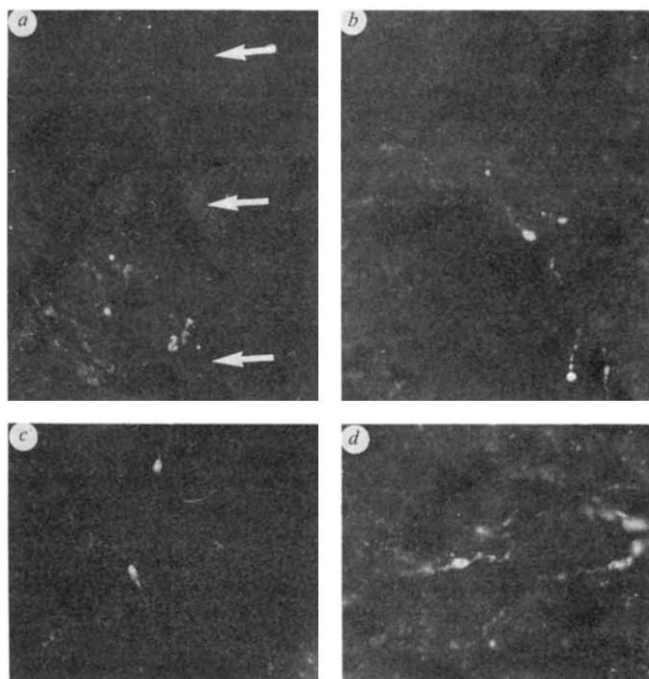


Fig. 2 a, Build-up of dynorphin B immunoreactivity in axons in the caudate nucleus proximal to a knife cut separating the globus pallidus from the striatum (arrow heads). $\times 75$. b-d, Dynorphin B immunoreactive cell bodies in the head of the caudate nucleus. $\times 350$.

Methods: Visualization of the dynorphin B immunoreactivity in brain tissue was carried out using the indirect fluorescent immunocytochemical technique²⁸. The animals received an intraventricular injection of colchicine 24 h before they were anaesthetized with chloral hydrate and perfused with 4% phosphate-buffered paraformaldehyde solution. After a 90-min post-fixation the brains were washed in 0.1 M phosphate buffer overnight and then frozen on a liquid CO₂ stage. Coronal sections (10 μ m thick) were cut in a microtome cryostat and mounted on gelatin-coated slides. The primary antibody, at a final dilution of 1:600, was incubated with the sections overnight at 4°C. The sections were then incubated with rhodamine labelled secondary antibody (1:10) for 30 min at 37°C. The slides were mounted in phosphate-buffered saline/glycerol and examined with a Zeiss microscope with epifluorescent illumination. There was no staining when the antibody was preabsorbed with an excess of dynorphin B.

Our data show that dynorphin-producing cells in the head of the caudate send fibres to the globus pallidus and substantia nigra. We cannot rule out the possibility that proenkephalin and prodynorphin coexist in the pallidal fibre population, but in the substantia nigra this does not seem to be the case, otherwise Met-enkephalin-Arg-Gly-Leu levels would decrease after striatonigral lesions, as do levels of α -neo-endorphin and dynorphin B. The most parsimonious explanation for the parallel decreases in α -neo-endorphin, dynorphin B and Leu-enkephalin is that in the striatonigral pathway Leu-enkephalin is made by dynorphinergic neurones.

Prodynorphin-derived peptides are potent κ -opiate receptor agonists²¹ while Leu-enkephalin is a δ -opiate receptor agonist²¹. Thus, the same precursor (prodynorphin) may yield ligands for different opiate receptor subtypes that can exert different actions. The fact that Leu-enkephalin is derived from two separate precursors in brain may explain, in part, the different Leu:Met-enkephalin ratios found in various brain regions.

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- Hughes, J. *et al.* *Nature* **258**, 577-579 (1975).
- Maysinger, D. *et al.* *Neuropeptides* **2**, 211-226 (1982).
- North, R. A. & Egan, T. M. *Br. med. Bull.* **39**, 71-76 (1983).
- Udenfriend, S. & Kilpatrick, D. L. *Archs Biochem. Biophys.* **221**, 309-323 (1983).
- Kilpatrick, D. L., Wahlstrom, A., Lhm, H. W., Blacher, R. & Udenfriend, S. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6480-6483 (1982).
- Kangawa, K., Minamino, N., Chino, N., Sakakibara, S. & Matsuo, H. *Biochem. biophys. Res. Commun.* **99**, 871-878 (1981).
- Goldstein, A., Fischli, W., Lowney, L. I., Hunkapiller, M. & Hood, L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7219-7223 (1981).
- Minamino, N., Kangawa, K., Chino, N., Sakakibara, S. & Matsuo, H. *Biochem. biophys. Res. Commun.* **99**, 864-870 (1981).
- Noda, M. *et al.* *Nature* **295**, 202-208 (1982).
- Gubler, U., Seeburg, P., Hoffman, B. J., Gage, L. P. & Udenfriend, S. *Nature* **295**, 206-208 (1982).
- Comb, M., Seeburg, P. H., Adelman, J., Eiden, L. & Herbert, E. *Nature* **295**, 663-666 (1982).
- Kakidani, H. *et al.* *Nature* **298**, 245-249 (1982).
- Denis, D., Day, R. & Lemaire, S. *Int. J. Peptide Protein Res.* **19**, 18-24 (1982).
- Hughes, J. *Br. med. Bull.* **39**, 17-24 (1983).
- Zamir, N., Palkovits, M. & Brownstein, M. J. *Brain Res.* **280**, 81-93 (1983).
- Zamir, N., Palkovits, M., Weber, E. & Brownstein, M. J. *Brain Res.* (in the press).
- Cuello, A. C. & Paxinos, G. *Nature* **271**, 178-180 (1978).
- Vincent, S. R., Hökfelt, T., Christensson, I. & Terenius, L. *Neurosci. Lett.* **33**, 185-190 (1982).
- Vincent, S. R., Hökfelt, T., Christensson, I. & Terenius, L. *Eur. J. Pharmac.* **85**, 251-252 (1982).
- Palkovits, M., Brownstein, M. J. & Zamir, N. *Neuropeptides* (in the press).
- Paterson, S. J., Robson, L. E. & Kosterlitz, H. W. *Br. med. Bull.* **39**, 25-30 (1983).
- Palkovits, M. *Brain Res.* **59**, 449-450 (1973).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265-275 (1951).
- Weber, E. & Barchas, J. D. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1125-1129 (1983).
- Weber, E., Geis, R., Voigt, K. M. & Barchas, J. D. *Brain Res.* **260**, 166-171 (1983).
- Weber, E., Roth, K. A., Evan, C. J., Chang, J.-K. & Barchas, J. D. *Life Sci.* **31**, 1761-1764 (1982).
- Halasz, B. & Pupp, L. *Endocrinology* **77**, 553-562 (1965).
- Coons, A. H. in *General Cytochemical Methods* (ed. Danielli, J. F.) 399-422 (Academic, New York, 1958).

Dual role for *Dictyostelium* contact site B in phagocytosis and developmental size regulation

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Three cohesion molecules have been discovered in the slime mould *Dictyostelium discoideum*¹⁻⁴. Two of these molecules are involved in morphogenesis at the aggregation stage of the life cycle and thereafter, and may also provide an essential trigger for late gene expression⁵. The third glycoprotein 126 (gp126) or contact site B, is present on axenically grown vegetative amoebae and persists to the aggregation stage where it is involved in the side-to-side cohesion of cells in aggregation streams^{1,4}. It is puzzling that vegetative amoebae should possess a cohesion molecule because their solitary habit does not necessitate cohesion. However, they do need to adhere to the substratum and adhesion of bacteria to the cells is a prerequisite for phagocytosis. Vogel *et al.*⁶ have proposed that the same receptor is involved in phagocytosis and cohesion. It has also been suggested that contact site B-mediated cohesion is a trigger for development⁷. Using a specific antibody against gp126, we now show that contact site B is a phagocytosis receptor. Furthermore, contact site B is involved in regulating the size of aggregates formed during morphogenesis; it also seems to be involved in cell-substratum adhesion but is not a developmental trigger.

A rabbit antibody (designated CMC13) was raised against gp126 purified to homogeneity by SDS-polyacrylamide gel electrophoresis. gp126 was the only cell surface protein to be immunoprecipitated by this antibody from vegetative cells of strain Ax2 (Fig. 1a). (Previously, gp126 was termed p126, but it has been shown to be glycosylated by periodic acid-dansyl hydrazine staining⁸.)

The first question investigated using this antibody was whether gp126 is a phagocytosis receptor. A phagocytosis assay was carried out essentially according to Glynn⁹ using ¹⁴C-lysine-labelled *Escherichia coli* strain B/r. Univalent Fab' fragments were prepared from both CMC13 and a control antibody, designated N. CMC13 Fab' completely inhibits cell cohesion at a concentration of 0.7 mg ml⁻¹. Fab' N provides a suitable control because it binds to the cell surface but does not block cohesion⁴. In the phagocytosis assay (Fig. 2) CMC13 blocked phagocytosis at 0.1 mg ml⁻¹ whereas Fab' N had no effect on phagocytosis at eight times this concentration. We conclude that gp126 is the phagocytosis receptor as well as being the vegetative cohesion molecule. This conclusion offers an explanation for the anomaly that vegetative axenic *D. discoideum* cells are cohesive¹⁰, whereas vegetative cells grown on bacteria are non-cohesive (ref. 11 and our unpublished observations). Thus, cells involved in phagocytosis could be depleted of cell surface receptor molecules because of receptor internalization whereas axenic cells would have a full complement of receptor molecules on their surfaces. To test this, we immunoprecipitated extracts of axenically grown Ax2 cells, bacterially grown Ax2 cells and NC-4 (wild type) cells. Figure 1b shows that gp126 is dramatically reduced in bacterially grown cells. This confirms the view that the cohesion receptor and the bacterial receptor are the same.

To determine whether gp126 is also involved in cell-substratum adhesion log phase axenic cells were suspended at 2×10^4 ml⁻¹ in Bonner's solution containing 1 mg ml⁻¹ of inhibi-

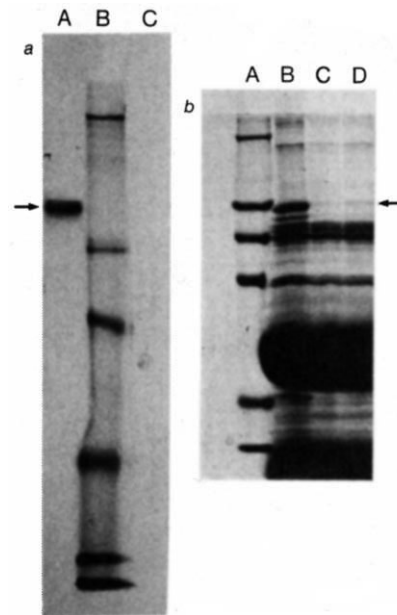


Fig. 1 a, Autoradiograph showing gp126 immunoprecipitated by CMC 13 IgG. Vegetative Ax2 cells were radioiodinated and a detergent extract of the cells was immunoprecipitated with 150 µg CMC 13 IgG, using methods described elsewhere⁴. Lane A, ¹²⁵I-gp126 immunoprecipitated by CMC 13; B, ¹⁴C-molecular weight (MW) markers (200,000, 92,000, 69,000, 46,000 and 30,000); C, pre-injection IgG. The same polypeptide (gp126) is the only Coomassie blue-stained band in immunoprecipitates of whole cells. b, gp126 depleted in cells grown on *E. coli* B/r. Detergent extracts of 5×10^7 cells were immunoprecipitated by the anti-vegetative cell antiserum CMC 7 (see ref. 4). Lane A, MW markers (200,000, 130,000, 97,400, 66,000, 45,000 and 29,000); B, axenic Ax2 cells; C, Ax2 cells grown on *E. coli* B/r; D, NC4 cells grown on *E. coli* B/r. The arrow denotes gp126.

tory Fab'. They were then allowed to settle onto a coverslip for 30 min in the presence of Fab', after which time the slide was inverted and the percentage of cells remaining attached after a further 30 min determined. Controls used Fab' N and Bonner's solution alone. The percentage of cells remaining attached for the various treatments was: Bonner's solution, 94%; Fab' CMC 13 (1 mg ml⁻¹), 13%; Fab' N (1 mg ml⁻¹), 95%. Therefore, inhibitory Fab' greatly reduces adhesion of cells to a glass substratum.

Anti-gp126 Fab' has allowed us to test whether contact site B is a trigger for development⁷. Cells incubated in suspension in the presence of inhibitory Fab' (1 mg ml⁻¹) became aggregation-competent in 7–8 h. They did not cohere initially but by 7 h had developed the EDTA-resistant cohesion characteristic of the aggregation stage of the life cycle^{1,2}. All cells possessed stainable Fab' on their surfaces after 7 h and the cell-free supernatant was still able to inhibit the cohesion of vegetative cells. Cells incubated on Millipore filters in the presence of inhibitory Fab' developed synchronously to the fruiting body stage in 24 h as did control cultures; the only difference was that grexes and fruiting bodies formed in the presence of inhibitory Fab' were much smaller than those in control cultures (Fig. 3). This difference in morphology was similar to that shown by developing Ax2 cells grown in the absence and presence of glucose¹². Thus, gp126 makes a significant contribution to the cohesiveness of developing cells and has a role in size regulation. Blocking of gp126 with inhibitory Fab' reduces cellular cohesiveness and thereby reduces aggregate, and hence fruiting body, size. Development in the presence of inhibitory IgG was entirely normal, presumably because capping results in its removal from the cell surface. Because cells can develop in conditions in which

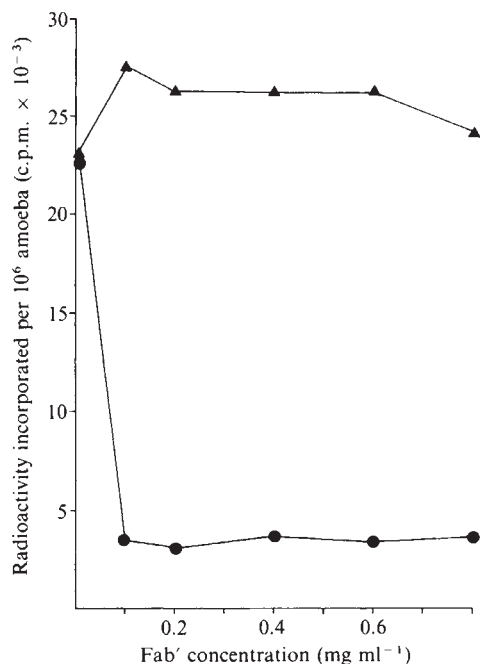


Fig. 2 Phagocytosis of ^{14}C -labelled *E. coli* B/r. $\blacktriangle$, Fab' N; $\bullet$, CMC 13 Fab'. *E. coli* B/r was labelled by growth overnight in 100 ml of a shaken (140 r.p.m., 35 °C) solution of 0.05% sodium citrate, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 , 0.7% K_2HPO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.27% glucose containing $0.25 \mu\text{Ci ml}^{-1}$ L-[U- ^{14}C]lysine (Amersham). Bacteria were washed in 17 mM Na/K phosphate buffer, and suspended in buffer at $2 \times 10^9 \text{ ml}^{-1}$. Log phase axenic amoebae were washed in phosphate buffer and suspended in buffer at $2 \times 10^6 \text{ cells ml}^{-1}$. Samples (0.5 ml) of amoebae were mixed with 1 ml phosphate buffer containing Fab' at the appropriate concentration. The Fab' was allowed to bind to the cells for 10 min, then 0.5 ml of *E. coli* was added and the resulting suspension shaken for 90 min at 140 r.p.m., 22 °C. Phagocytosis was stopped with 2 ml of 4% glutaraldehyde. After 5 min of fixation, 5×10^7 stationary phase Ax2 cells were added as a carrier. Cells were collected by bench centrifugation then washed twice in 17 mM Na/K phosphate buffer to remove unbound *E. coli*. The pellet of washed cells was dissolved in 2 ml of scintillant (750 ml Triton X-100, 1,500 ml toluene, 6 g 2,5-diphenyloxazole). The radioactivity was measured with a Packard Tri Carb 300 β -counter. The specific radioactivity of the *E. coli* suspension was 34,500 c.p.m. per 10^8 bacteria. As a maximum of 27,000 c.p.m. was incorporated into 10^6 amoebae (in the presence of Fab' N), each amoeba bound an average of 78 bacteria in permissive conditions. Glynn⁹ obtained a value of 80 bacteria per cell.

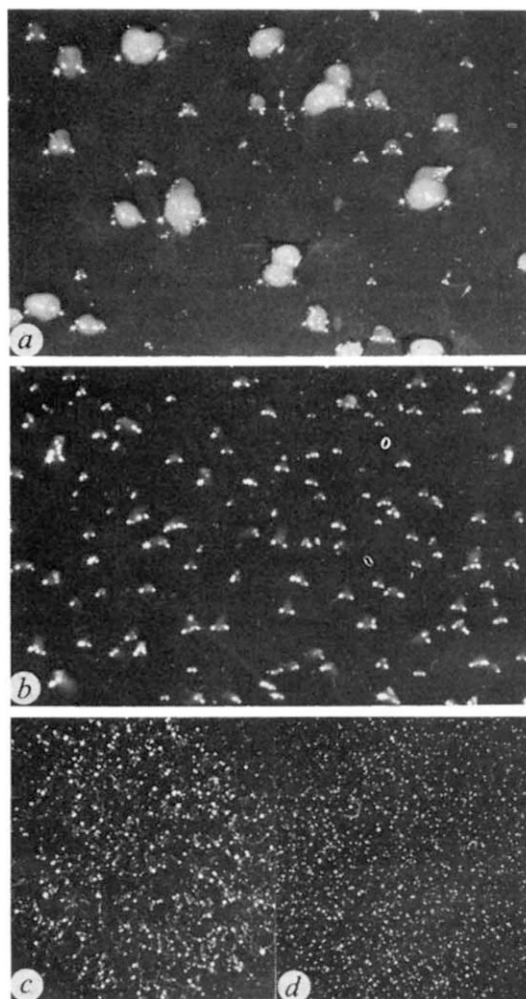


Fig. 3 Size regulation. *a*, Control grexes in Fab' N (18 h). ($\times 6.5$.) *b*, Grexes in inhibitory Fab' (18 h). ($\times 6.5$.) *c*, Control fruiting bodies in Fab' N (24 h). ($\times 1.2$.) *d*, Fruiting bodies in inhibitory Fab' (24 h). ($\times 1.2$.) Axenically grown cells were allowed to develop on Millipore filters according to the method of Sussman¹³. Inhibitory Fab' (1.5 mg ml^{-1}) was added to the culture solution. The cells were allowed to develop for 24 h at 22 °C and were observed at intervals.

gp126-mediated cohesion is blocked, this molecule cannot be a developmental trigger.

Thus, the *Dictyostelium* cohesion molecule known as contact site B or gp126 has a dual role in phagocytosis and morphogenetic size regulation. We speculate that this is of evolutionary significance since the development of receptor-mediated phagocytosis could have provided the basis for cell-cell adhesion

and hence the multicellularity displayed by higher organisms. It is possible that gp126 is related to the nonspecific receptor postulated by Vogel *et al.*⁶, because it also seems to be involved in cell adhesion to glass.

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1. Beug, H., Gerisch, G., Kempff, S., Riedel, B. & Cremer, G. *Expl Cell Res.* **63**, 147-158 (1970).
2. Beug, H., Katz, F. E. & Gerisch, G. *J. Cell Biol.* **56**, 647-658 (1973).
3. Steinemann, C. & Parish, R. W. *Nature* **286**, 621-623 (1980).
4. Chadwick, C. M. & Garrod, D. R. *J. Cell Sci.* **60**, 251-266 (1983).
5. Mangiarotti, G., Ceccarelli, A. & Lodish, H. F. *Nature* **301**, 616-618 (1983).
6. Vogel, G., Thilo, L., Schwarz, H. & Steinhart, R. *J. Cell Biol.* **86**, 456-465 (1980).

7. Marin, F. T., Goyette-Boulay, M. & Rothman, F. G. *Devl Biol.* **80**, 301-312 (1980).
8. Eckhardt, A. E., Hayes, C. E. & Goldstein, L. J. *Analyt. Biochem.* **73**, 192-197 (1976).
9. Glynn, P. J. *Cytobios* **30**, 153-166 (1981).
10. Swan, A. P. & Garrod, D. R. *Expl Cell Res.* **93**, 479-484 (1975).
11. Garrod, D. R. *Expl Cell Res.* **72**, 588-591 (1972).
12. Garrod, D. R. & Ashworth, J. M. *J. Embryol. exp. Morph.* **28**, 463-479 (1972).
13. Sussman, M. in *Methods in Cell Physiology* Vol. 2, Ch. 14, 397-410 (Academic, New York, 1966).

Induction of human vascular endothelial stress fibres by fluid shear stress

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Endothelial cells of the arterial vascular system and the heart contain straight actin filament bundles, of which there are few, if any, in the venous endothelium¹⁻⁴. Since stress fibre-containing endothelial cells within the vascular system tend to be located at sites exposed to particularly high shear stress of blood flow, we have investigated, in an experimental rheological system (Fig. 1), the response of the endothelial actin filament skeleton to controlled levels of fluid shear stress. Here we report that endothelial stress fibres can be induced by a 3-h exposure of confluent monolayer cultures of human vascular endothelium to a fluid shear stress of 2 dynes cm⁻², approximately the stress occurring in human arteries *in vivo*. Fourfold lower levels of shear stress that normally occur only in veins, had no significant effect on the endothelial actin filament system. The formation of endothelial stress fibres in response to critical levels of fluid shear stress is probably a functionally important mechanism that protects the endothelium from hydrodynamic injury and detachment.

Endothelial cells were collected from human umbilical vein⁵ and grown on glass coverslips previously coated with a layer of extracellular matrix⁶. This coating (achieved by precultivation of the coverslips with bovine corneal endothelial cells) was important in that it allowed the formation of confluent human endothelial monolayer cultures that were firmly attached to the substrate (Fig. 2). Cells grown on glass were generally less firmly attached to the surface as revealed by occasional detachment of individual cells and even large groups of cells during exposure to fluid shear stress. Fluorescent staining of the confluent cultures with rhodamine-labelled phalloidin (a specific probe for polymerized actin⁷) and with antibodies to calf thymus myosin² and chicken gizzard and pectoral muscle α -actinin⁸ showed a rather uniform pattern of stress fibres (Fig. 3). The stress fibres displayed the typical continuous staining for actin and an interrupted pattern for myosin and α -actinin similar to the descriptions of stress fibres found in cultured aortic endothelium^{1,9} and other tissue culture cells (for a recent review, see refs 10, 11). Stress fibre staining was most prominent in the peripheral cytoplasm close to the margin of the polygonally-shaped cells. We often observed intensely stained focal areas from which numerous stress fibres were seen to radiate in various directions. Outside the marginal zone intensely stained stress fibres were rather infrequent. Most of the relatively few stress fibres projecting over the central parts of the cells appeared to be rather thin and were only faintly stained.

Controlled levels of shear stress were then applied by using a transparent rotating cone placed with its tip on the centre of the coverslips (Fig. 1). Rotation of the cone caused the culture medium to flow between the cone and the surface of the coverslip. In this study a lamina flow was chosen, producing a shear stress of 2 dynes cm⁻², which is about the minimum level in human arteries for basal conditions (2–20 dynes cm⁻², locally

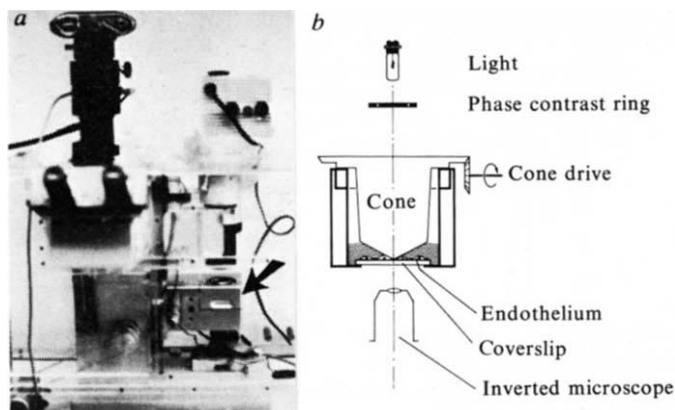


Fig. 1 *a*, Rheoscope used in this study. The construction is based on a cone and plate mechanism originally developed for microrheological studies of blood cell aggregations²⁰. An arrow indicates the rheoscope chamber, which is shown in *b* in simplified cross-sectional form (not to scale). A transparent polycarbonate cone having an angle of 2.5° is fitted into a Plexiglas holder and connected to a speed-controlled motor with variable rotational velocities. The cone is placed with its tip on the surface of the coverslip under microscopic control. The direction and magnitude of shear stress were determined precisely both by direct measurements and from theory (ref. 21 and our unpublished data).

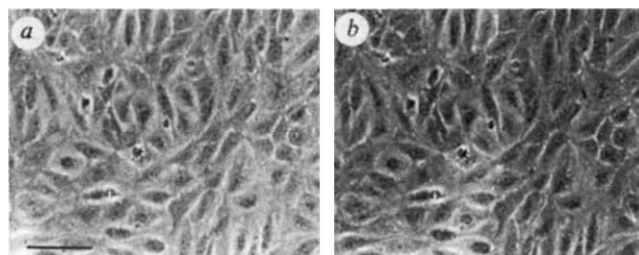


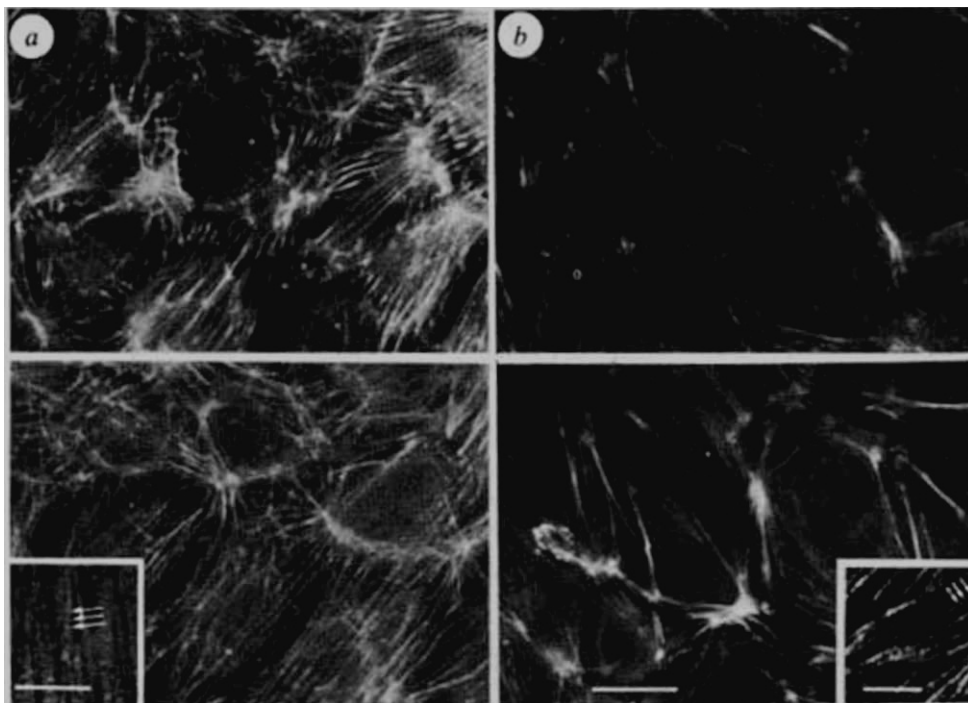
Fig. 2 *a, b*, Phase contrast micrographs of confluent monolayer cultures of human vascular endothelial cells collected from the umbilical vein⁵, cultured in medium TC 199 containing 20% pooled human serum and 1 mg ml⁻¹ endothelial growth supplement extracted from bovine brain²². Cells were seeded on coverslips previously coated with an extracellular matrix layer consisting of bovine corneal endothelium⁶, and were grown for 8–10 days. The same area of the monolayer was monitored throughout the exposure to fluid shear stress (2 dynes cm⁻²). *a*, Before shear stress exposure; *b*, after 3 h of exposure. No changes in cellular shape or position have occurred. Scale bar, 100 μ m.

up to 100 dynes cm⁻²)¹². No changes in cellular shape or position occurred during exposure to shear stress, as judged by phase contrast microscopy and TV recording of the endothelium throughout the exposure period (1, 2, 3 and 4 h). Temperature and pH of the medium were kept constant during the experiments.

After a 3-h exposure to shear stress, the quantity and staining intensity of stress fibres had dramatically increased (Fig. 3). No preferential alignment or direction of the newly formed stress fibres was seen. The exact time course of induction of stress fibres has so far not been determined. At present we can only state that after a 1-h exposure to shear stress there was no increase in the amount of stress fibres, but a slight increase was observed after 2 h. After 4 h the quantity and density of stress fibres were about the same as after 3 h of exposure to shear stress. Thus most stress fibres seemed to have developed during the second and third hour of shear stress treatment. When the shear stress was reduced to fourfold lower levels (0.5 dynes cm⁻²), a level normally found only in the venous system, no significant increase in the quantity of stress fibres was seen after 3 h of exposure.

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Fig. 3 *a*, Pattern of actin filament bundles (stress fibres) in human endothelial monolayer cultures (two examples are shown) after 3 h of exposure to low arterial levels of fluid shear stress (2 dynes cm^{-2}). *b*, Corresponding controls. Scale bar, $20 \mu\text{m}$. Only the central parts of the monolayers (area of $\sim 1 \text{ cm}^2$) were examined, because endothelial cells located close to the margin of the coverslips were still in a subconfluent state and thus contained varying amounts of stress fibres. Each shear stress experiment was followed by a control experiment in which corresponding cultures were placed in the rheoscope chamber for the same length of time as for exposure to shear stress, but without rotation of the cone. Stress fibres were visualized by fluorescent staining with $1.4 \mu\text{g ml}^{-1}$ tetramethylrhodaminyl- which was applied to the monolayers after 5 min of fixation with 2% paraformaldehyde in phosphate-buffered saline and 3 min of permeabilization with 80% acetone (all steps were done at room temperature). The insets show stress fibres at higher magnification (scale bars, $10 \mu\text{m}$) stained with antibodies to calf thymus myosin (inset in *a*) and chicken gizzard α -actinin (inset in *b*) using the indirect immunofluorescence technique described elsewhere².



Although no attempts were made to determine the threshold value of shear stress that induces stress fibre formation in this *in vitro* system, the experiments reported here clearly show that at a critical level of fluid shear force (which seems to be much lower *in vitro* than *in situ*) endothelial cells will respond by forming stress fibres. These stress fibres probably protect the cells against the shear stress of fluid flow. In view of the observation that isolated stress fibres can contract in appropriate conditions^{13,14}, it seems reasonable to assume that endothelial stress fibres may apply tension to resist the shear forces acting on the cells, thus allowing the cells to maintain their flattened phenotype and to remain firmly attached to the substratum. To a certain degree, stress fibre formation may prevent hydrodynamic damage to the endothelium and thus protect the vascular wall from certain pathological stimuli such as those thought to be involved in the initiation of arteriosclerosis¹⁵.

The shear-induced stress fibres were more or less randomly distributed with respect to the direction of fluid flow. This pattern apparently differs from the alignment of stress fibres in arterial endothelial cells *in situ*, which is characterized by orientation of the fibres parallel to the direction of blood flow^{1-4,16}. However, as the arterial endothelium consists of elongated cells having their long axis oriented in the direction of blood flow (this kind of orientation is probably caused by long-term shear stress in one direction)¹⁷, and in view of the fact that in elongated, cultured cells stress fibres are mainly aligned parallel to the cellular long axis¹⁸, it seems likely that the uniform alignment of arterial endothelial stress fibres parallel to the blood flow depends on the particular orientation of arterial endothelial cells rather than on direct actions of fluid shear stress on the orientation of stress fibres. In the experimental conditions of the present study, the confluent polygonal endothelial cells did not change their shape during shear stress; this is probably the reason why the induced stress fibres did not display a uniform alignment in the direction of the fluid flow. Support for this view comes from regenerating arterial endothelial cells of the rabbit aorta: at the edges of the outgrowing endothelium, stress fibres were frequently found oriented diagonally or even perpendicularly to the blood flow but the fibres were always aligned parallel to the cellular long axis³. Similar observations were obtained with subconfluent and con-

fluent cultures of endothelium exposed to long-term (1–2 days) shear stress. Reorientation of endothelial cells in the direction of the fluid flow was accompanied by re-alignment of the stress fibres which were always (before and after the shear treatment) oriented roughly parallel to the cellular long axis (ref. 19 and our unpublished observations). However, such changes in the alignment of stress fibres seem to be only secondary effects brought about by shear stress-induced reorientation of endothelial cells.

The present study provides the first evidence that in human vascular endothelium, fluid shear stress may act directly on the stress fibre system without affecting cellular shape and orientation. The factors mediating this response of the endothelial actin filament system remain to be determined.

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1. Drenckhahn, D. *Prog. appl. Microcirculation* **1**, 53–70 (1983).
2. Drenckhahn, D., Gröschel-Stewart, U., Kendrick-Jones, J. & Scholey, J. *Eur. J. Cell Biol.* **30**, 100–111 (1983).
3. Gabbiani, G., Gabbiani, F., Lombardi, D. & Schwartz, S. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2361–2364 (1983).
4. Wong, A. J., Pollard, T. D. & Herman, I. *Science* **219**, 867–869 (1983).
5. Jaffé, E. A., Nachman, R. L., Becker, D. G. & Minick, C. R. *J. clin. Invest.* **52**, 2745–2756 (1973).
6. Gospodarowicz, D., Vlodavsky, J. & Savion, N. *J. supramolec. Struct.* **13**, 339–372 (1980).
7. Faulstich, H., Trischmann, H. & Mayer, D. *Expl Cell Res.* **144**, 73–82 (1983).
8. Drenckhahn, D. & Mannherz, H. *Eur. J. Cell Biol.* **30**, 167–176 (1983).
9. Kalnins, V. I. & Subrahmanyam, L. *Eur. J. Cell Biol.* **24**, 36–44 (1981).
10. Goldman, R. D., Milsted, A., Schloss, J. A., Starger, J. & Yerna, M. J. *A. Rev. Physiol.* **41**, 703–722 (1979).
11. Gröschel-Stewart, U. & Drenckhahn, D. *Collagen Rel. Res.* **2**, 381–463 (1982).
12. Dewey, C. F., Bussolari, S. R., Gimbrone, M. A. & Davies, P. F. *J. biomech. Eng.* **103**, 177–185 (1981).
13. Isenberg, G., Rathke, P. C., Hülsmann, N., Franke, W. W. & Wohlfarth-Bottermann, K. E. *Cell Tissue Res.* **166**, 427–428 (1976).
14. Kreis, T. E. & Birchmeyer, W. *Cell* **22**, 555–561 (1980).
15. Ross, R. & Glomset, J. A. *New Engl. J. Med.* **295**, 369–377, 420–425 (1976).
16. Hammersen, F. *Adv. Microcirculation* **9**, 95–134 (1980).
17. Langille, B. L. & Adamson, S. L. *Circulation Res.* **48**, 481–488 (1981).
18. Buckley, I. K. & Porter, K. *Protoplasma* **64**, 350–380 (1967).
19. White, G. E., Fujiwara, K., Shefton, E., Dewey, C. F. & Gimbrone, M. A. *Fedn Proc.* **41**, 321 (Abstr.) (1982).
20. Schmidt-Schönbein, H., Gosen, J. V., Heinrich, L., Klose, H. J. & Vogeler, E. *Microvasc. Res.* **6**, 366–376 (1973).
21. Kiesewetter, H. *et al. Biomed. Techn.* **27**, 209–213 (1982).
22. Maciag, T., Cerundolo, J., Ilfley, S., Kelley, P. R. & Forand, R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5674–5678 (1979).

Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues

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Chondroitin sulphate proteoglycans are widespread connective tissue components¹⁻⁴ and chemical analysis of cartilage and other proteoglycans has demonstrated molecular speciation involving the degree and position of sulphation of the carbohydrate chains^{5,6}. This may, in turn, affect the properties of the glycosaminoglycan (GAG), particularly with respect to self-association and interactions with other extracellular matrix components^{6,7}. Interactions with specific molecules from different connective tissue types, such as the collagens^{8,9} and their associated glycoproteins^{10,11}, could be favoured by particular charge organizations on the GAG molecule endowed by the sulphate groups. So far, it has not been possible to identify and map chondroitins of differing sulphation in tissues, but we have now raised three monoclonal antibodies which specifically recognize unsulphated, 4-sulphated and 6-sulphated chondroitin and dermatan sulphate. These provide novel opportunities to study the *in vivo* distribution of chondroitin sulphate proteoglycans. We demonstrate that chondroitin sulphates exhibit remarkable connective tissue specificity and furthermore provide evidence that some proteoglycans may predominantly carry only one type of chondroitin sulphate chain.

The characteristics, specificity and designation of each antibody are given in Table 1 and Fig. 1. Details of the methodology and protocols used are given elsewhere¹². Rat tissue sections for indirect immunofluorescence staining with these antibodies required pretreatment with GAG-degrading enzymes to liberate the specific antigen as listed in Table 1. The omission of enzyme treatment in the staining schedule served as a control revealing nonspecific staining and was negative for each monoclonal antibody. Figure 1c shows that 3-B-3 antibody has minor activity against unsulphated chondroitin (C0-S) in addition to the major determinant, chondroitin 6-sulphate (C6-S). However, the comparative use of both the 1-B-5 and 3-B-3 antibodies allows the distribution of C0-S and C6-S to be easily determined.

In the rat we found that the distribution of C0-S was virtually restricted to cartilage (Fig. 2a) since the 1-B-5 antibody stained articular and hyaline cartilage brightly, but this antibody did not stain dermis or other connective tissues apart from a faint staining in the intima of rat aorta (Fig. 2b). However, chondroitin 4-sulphate (C4-S) and/or dermatan sulphate (DS) was more widely distributed than unsulphated chondroitin. In addition to cartilage staining by the 9-A-2 antibody (Fig. 3a), fluorescence was widely distributed in loose connective tissue. The dermis, in particular, was well stained in a fibrillar pattern suggesting some association with dermal collagen fibres (Fig. 3b) as was the connective tissue supporting blood vessels, nerve fibres and striated muscle from a variety of locations (Fig. 3c). The media and adventitia of the aorta stained positively as did the kidney mesangium (Figs 3d, e). Epithelia and endothelial components of the skin (Fig. 3b), aorta (Fig. 3d) and kidney were themselves negative as were their supporting basement membrane zones (BMZ), including those of the glomerulus. The indications are that a BMZ population of C4-S or DS was not detectable. A further series of experiments was performed to establish whether some or all of the loose connective tissue staining was in fact chondroitin 4-sulphate (C4-S) or dermatan sulphate (DS). Accordingly, tissue sections were pretreated with

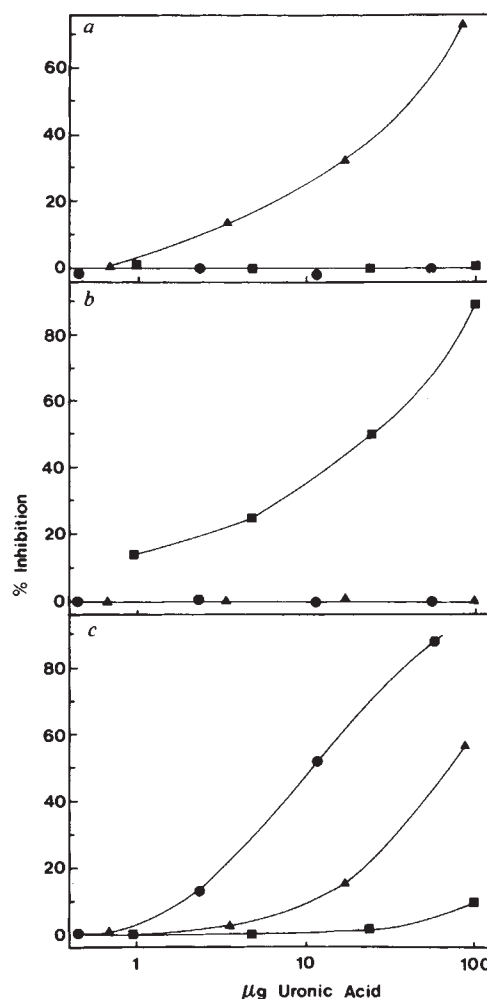


Fig. 1 Antibody characterization by inhibition of the binding of ¹²⁵I-labelled chondroitinase ABC-digested rat chondrosarcoma proteoglycan to monoclonal antibodies as a function of the concentration of chondroitin sulphate disaccharides. The disaccharides tested were 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulpho-D-galactose (■), the corresponding 6-sulphated disaccharide (●), and the unsulphated disaccharide (▲). ¹²⁵I-antigen (about 1 ng of protein containing 3 × 10⁴ c.p.m.) and chondroitin sulphate disaccharides in the amounts indicated were incubated with a, antibody 1-B-5 (1/400 dilution of ascites fluid), b, antibody 9-A-2 (1/100 dilution), or c, antibody 3-B-3 (1/400 dilution) in a total volume of 200 µl of buffer containing 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% bovine serum albumin, 0.15 M NaCl, 0.03% Na₂S₂O₅, 0.01 M sodium phosphate, pH 8.0. After incubation at room temperature (22–25 °C) for 90 min the antibody-bound antigen was separated from the unbound using heat- and formalin-inactivated *Staphylococcus aureus* cells as described previously³⁰. Per cent inhibition was calculated relative to the amount of ¹²⁵I-labelled antigen bound to the antibody in the absence of added chondroitin sulphate disaccharides. The conditions for use of the antibodies are shown in Table 1.

chondroitinase ACII in place of chondroitinase ABC. Under these conditions C4-S but not DS is digested¹³. We found that cartilage staining persisted (Fig. 3f) but the dermis of skin and other loose connective tissue was now weakly stained or unstained (Fig. 3g). We, therefore, deduce that the staining depicted in Figs 3a and b is largely derived from DS.

Chondroitin 6-sulphate (C6-S) distributions were revealed by the 3-B-3 antibody after chondroitinase ABC or testicular hyaluronidase pretreatment of tissue sections. In addition to cartilage staining (Fig. 4a), very heavy staining of the intima and media of the aorta was detected (Fig. 4c) as was a remarkable specificity for a number of BMZ in the rat including those of blood vessel endothelia, muscle, peripheral nerve fibres, the

dermal/epidermal junction, kidney tubules and the glomerulus (Figs 4a-f). Further tests were carried out by enzyme-linked immunosorbent assay and no cross-reactivity (not shown) between the antibody and other previously characterized BMZ components such as laminin¹⁴ and type IV collagen¹⁵ was detected. Antigen adsorption with rat liver heparan sulphate proteoglycan¹⁶ did not block BMZ staining. In addition, the tissue-staining patterns seen with two antisera against heparan sulphate proteoglycan^{17,18} were quite distinct from that with the 3-B-3 antibody (not shown). The presence of a BMZ population of C6-S proteoglycan has not previously been shown by immunohistochemical means, but chemical analysis of glomerular BMZ has indicated a minor amount of chondroitin sulphate in addition to the predominant GAG, heparan sulphate^{19,20}. The presence of a chondroitin sulphate proteoglycan in the supernatants of vascular endothelial cell cultures has recently been reported and is consistent with our results²¹. The precise localization of C6-S within BMZ is currently under investigation by electron microscopy.

Although the antigens for monoclonal antibody production were in each case of cartilage origin, the epitopes recognized by the antibodies are common to other connective tissue locations and are revealed by chondroitinase ABC pretreatment. The possibility that masking of other potentially antigenic sites occurs through the interaction with other components cannot be discounted but seems unlikely in view of the specificity of staining patterns shown here. Moreover, our findings agree in principle with previous chemical analyses of whole tissues. For example, chondroitin chains with differing sulphation (C0-S, C4-S and C6-S) have been reported from cartilage²², and we found that all three monoclonals stained both articular and hyaline cartilage intensely. Similarly, the predominant GAG types in aorta have been reported to be C6-S and DS²³, results entirely consistent with our immunofluorescence localizations. An advantage of our methods is, however, that we have shown that these two chondroitin subspecies do not share the same tissue locations (Figs 3, 4). While C6-S was present only in the intima and media, C4-S was prominent in the adventitia region and also the media but not the intima. This is just one example of the much higher resolution that can be achieved by immunohistochemical detection of glycosaminoglycans over previous methods. Some similarity in properties between cartilage and aorta proteoglycans have also been noted before^{24,25}. In contrast to cartilage it seems that for other tissue locations chondroitin sulphate chains of only one major type are covalently bound to a specific protein core. Other reports have

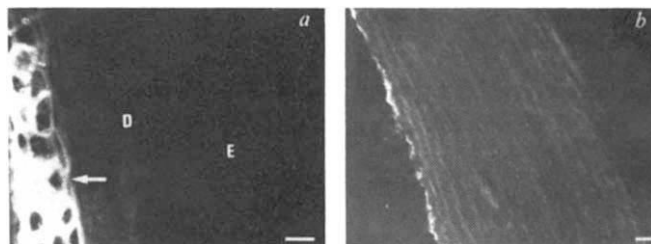


Fig. 2 1-B-5 antibody staining showing the distribution of unsulphated chondroitin in chondroitinase ABC treated (0.2 U ml^{-1} in 0.1 M Tris-acetate pH 7.3 containing 1 mg ml^{-1} bovine serum albumin) rat ear (a) and aorta (b) sections. Cartilage was heavily stained (arrow), but the epidermis (E) and dermis (D) with its associated muscle fibres, blood vessels and nerve fibres were unstained. The intima of the rat aorta was weakly positive with the 1-B-5 antibody. Scale bars, $15 \mu\text{m}$. For this and all other staining, small pieces of freshly excised tissue from rats were snap-frozen in isopentane chilled in liquid nitrogen. Cryostat sections ($6\text{--}8 \mu\text{m}$) were briefly air-dried, fixed for 15 min in 3% formaldehyde, then washed repeatedly in Dulbecco's phosphate-buffered saline, including one 15-min wash supplemented with 0.1 M ammonium chloride. Antibody staining was for 30–45 min at 37°C in a wet-box with several washes between applications.

suggested that chondroitin sulphates of different types may occur within a single chain²⁶. One possibility for future consideration in this regard is that the striking tissue specificity of chondroitin sulphates shown here may reside in the disaccharides adjacent to the protein core of the proteoglycan, these remaining after enzyme treatment. Mixed forms of chondroitin sulphate, if present in more distal regions of the chondroitin chains would then be preferentially removed by the enzyme before antibody application. Recently, we have characterized an additional monoclonal antibody which specifically recognizes keratan sulphate and which could also be used for immunohistochemical analyses by techniques similar to those reported here¹².

This work has two important implications. First, the different forms of chondroitin recognized by the three monoclonal antibodies have distinct and often separable distributions. Our observations further indicate that the proteoglycans involved may predominantly carry only one type of chondroitin chain with respect to sulphation. If these deductions are correct it is possible that the core proteins of the chondroitin proteoglycans are distinct gene products expressed in a tissue or cell-specific manner with a concomitant specificity of chondroitin sulphate

Table 1 Conditions for use of the antibodies

Monoclonal antibody designation	Antigen	Antibody source for staining	Antibody class and working dilution	Second antibody and working dilution	Antibody specificity
1-B-5	Chondroitinase ABC digested rat chondrosarcoma proteoglycan	Ascites fluid	IgG1 1:50	Affinity purified goat anti-mouse IgG1-FITC or TRITC 0.05 mg ml^{-1}	Unsaturated uronic acid* residue linked to <i>N</i> -acetylgalactosamine
9-A-2	Chondroitinase ABC digested bovine articular cartilage proteoglycan	Ascites fluid	IgG1 1:50	Affinity purified goat anti-mouse IgG1-FITC or TRITC 0.05 mg ml^{-1}	Unsaturated uronic acid* residue linked to <i>N</i> -acetylgalactosamine 4-sulphate
3-B-3	Chondroitinase ABC digested rat chondrosarcoma proteoglycan	Ascites fluid	IgM 1:50	Affinity purified goat anti-mouse IgM-FITC or TRITC 0.1 mg ml^{-1}	Unsaturated* or saturated uronic acid residue linked to <i>N</i> -acetylgalactosamine 6-sulphate Some activity against unsulphated derivative

FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

*These represent the residues from chondroitinase ABC digestion of chondroitin, chondroitin 4-sulphate and chondroitin 6-sulphate respectively.

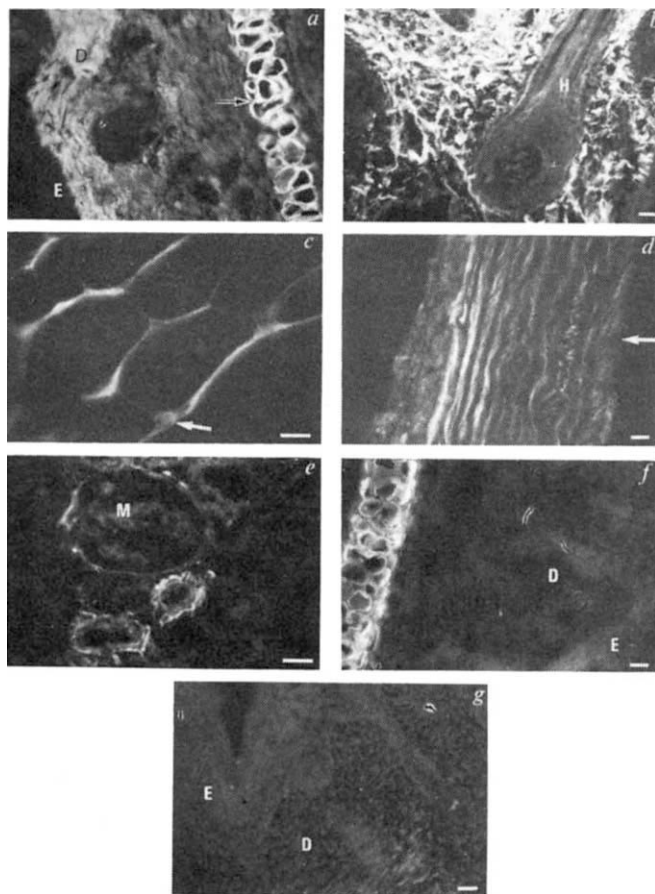


Fig. 3 9-A-2 antibody staining on rat tissue sections pretreated with chondroitinase ABC (a-e) to stain C4-S and DS, or chondroitinase AC II (f, g) to stain C4-S only¹². a, Ear cartilage (arrow) and dermal connective tissue (D) were heavily stained, but the epidermis (E) was negative. Dorsal rat skin dermis (b) had a fibrillar staining pattern and the epithelia of the hair shaft (H) were unstained. c, Connective tissue surrounding striated muscle and blood vessels (arrow) were also positively stained with this antibody. d, Staining was seen through most of the cross-section of rat aorta but no staining of the endothelial basement membrane zone was visible (arrow). e, Connective tissue surrounding kidney blood vessels and Bowman's capsule were positively stained together with weak staining of the mesangium (M). The tubular and glomerular basement membrane zones were apparently unstained. f, g, Sections of rat ear and dorsal skin respectively after chondroitinase AC II pretreatment (compare a and b). Only cartilage staining persisted indicating the presence of C4-S. Dermal connective tissue staining was lost indicating that it contained DS. Scale bars, 15 µm.

chains. Our data therefore provide further evidence for the specificity of connective tissue types, seen previously in terms of both collagenous^{15,27,28} and non-collagenous glycoproteins such as laminin¹⁴ and chondronectin²⁹. The specificity of sulphation of chondroitin sulphate proteoglycan in connective tissues may facilitate interactions with other matrix components.

We believe that these antibodies can be used to isolate and characterize the chondroitin sulphate proteoglycans and their protein cores from a variety of tissue sources. In particular, distinct protein cores can be isolated from mixed populations of proteoglycans; at present very little is known regarding the relationship between them and their interactions with other matrix components. Our preliminary findings also suggest that the antibodies have wide cross-species reactivity, as might be expected from the highly conserved nature of GAG chains. Antibodies such as these will therefore be valuable in the elucidation of the functions of chondroitin sulphate proteoglycans in

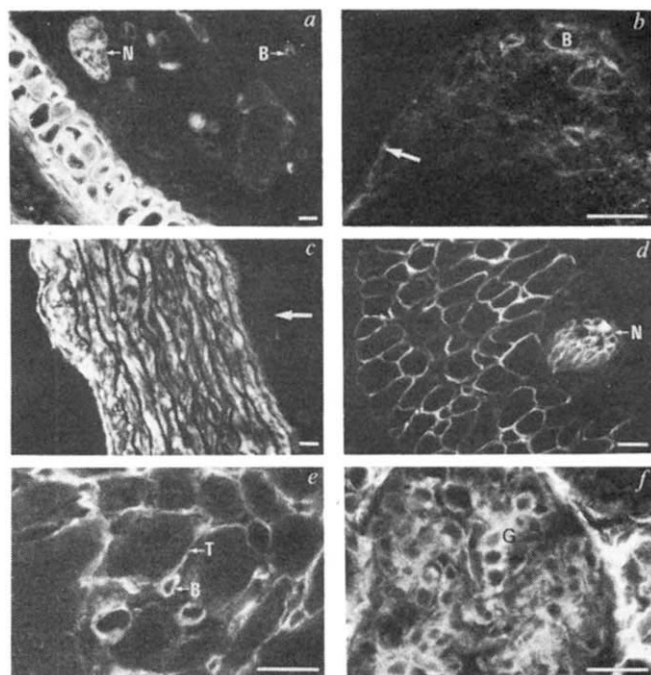


Fig. 4 Distribution of C6-S in chondroitinase ABC-treated rat tissue sections as revealed by the 3-B-3 antibody. In addition to ear cartilage (a), this antibody stained basement membrane zones of dermal nerve fibres (N) and blood vessels (B) (a, b), the dermo-epidermal junction (arrow) (b), intima and media of aorta but not adventitia (arrow) (c), basement membrane zones of striated muscle and associated nerve fibres (d) and those of kidney tubules (T) and glomerulus (G) (e, f). Scale bar, 15 µm.

human connective tissue synthesis and assembly as well as their roles in many pathological conditions.

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1. Muir, H. & Hardingham, T. E. *MTP Int. Rev. Sci. Biochem. Ser. 1* **5**, 153-222 (1975).
2. Dietrich, C. P., Sampaio, L. O. & Toledo, O. M. S. *Biochem. biophys. Res. Commun.* **71**, 1-10 (1976).
3. Hascall, V. C. & Hascall, G. K. in *Cell Biology of Extracellular Matrix* (ed. Hay, E. D.) 39-63 (Plenum, New York, 1981).
4. Caterson, B., Baker, J. R., Christner, J. E. & Couchman, J. R. *J. invest. Derm.* **79**, 45s-50s (1982).
5. Rodén, L. & Schwartz, N. B. *MTP Int. Rev. Sci. Biochem. Ser. 1* **5**, 96-152 (1975).
6. Lindahl, U. & Höök, M. A. *Rev. Biochem.* **47**, 385-417 (1978).
7. Fransson, L.-Å. *Biochim. biophys. Acta* **437**, 106-115 (1976).
8. Toole, B. P. *J. biol. Chem.* **251**, 895-897 (1976).
9. Scott, J. E. & Orford, C. R. *Biochem. J.* **197**, 213-216 (1981).
10. Ruoslahti, E. & Engvall, E. *Biochim. biophys. Acta* **631**, 350-358 (1980).
11. Del Rosso, M., Cappelletti, R., Viti, M., Vannucchi, S. & Chiarugi, V. *Biochem. J.* **199**, 699-704 (1981).
12. Caterson, B., Christner, J. E. & Baker, J. R. *J. biol. Chem.* **258**, 8848-8856 (1983).
13. Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. *J. biol. Chem.* **243**, 1523-1535 (1968).
14. Timpl, R. *et al. J. biol. Chem.* **254**, 9933-9937 (1979).
15. Kefalides, N. A., Alper, R. & Clark, C. C. *Int. Rev. Cytol.* **61**, 167-228 (1979).
16. Kjellén, L., Pettersson, I. & Höök, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5371-5375 (1981).
17. Hassell, J. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 4494-4498 (1980).
18. Woods, A., Kjellén, L., Höök, M., Smith, C. G. & Rees, D. A. *J. Cell biol.* (in the press).
19. Kanwar, Y. S., Hascall, V. C. & Farquhar, M. G. *J. Cell Biol.* **90**, 527-532 (1981).
20. Parthasarathy, N. & Spiro, R. G. *J. biol. Chem.* **256**, 507-513 (1981).
21. Oohira, A., Wight, T. N. & Bornstein, P. *J. biol. Chem.* **258**, 2014-2021 (1983).
22. Rodén, L., Baker, J. R., Cifonelli, J. A. & Mathews, M. B. *Meth. Enzym.* **28**, 73-140 (1972).
23. Toledo, O. M. S. & Dietrich, C. P. *Biochim. biophys. Acta* **498**, 114-122 (1979).
24. Antonopoulos, C. A., Axelsson, I., Heinegård, D. & Gardell, S. *Biochim. biophys. Acta* **338**, 108-119 (1974).
25. Gardell, S., Baker, J. R., Caterson, B., Heinegård, D. & Rodén, L. *Biochem. biophys. Res. Commun.* **95**, 1823-1831 (1980).
26. Seno, N., Akiyama, F. & Anno, K. *Biochim. biophys. Acta* **362**, 290-298 (1974).
27. Linsenmayer, T. F. in *Cell biology of Extracellular Matrix* (ed. Hay, E. D.) 5-37 (Plenum, New York, 1981).
28. Martinez-Hernandez, A., Gay, S. & Miller, E. J. *J. Cell Biol.* **92**, 343-349 (1982).
29. Hewitt, A. T. *et al. J. biol. Chem.* **257**, 2330-2334 (1982).
30. Christner, J. E., Caterson, B. & Baker, J. R. *J. biol. Chem.* **255**, 7102-7105 (1980).

Is stepwise sarcomere shortening an artefact?

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A report¹ in 1977 raised the intriguing possibility that sarcomere shortening in muscle may occur in a stepwise fashion, in which episodes of shortening are interrupted by periods of little or no movement. This was taken by its authors to imply the synchronous activity of cross-bridges over a large volume of tissue—behaviour which cannot easily be reconciled with commonly accepted views of muscle contraction^{2–5}. Stepwise shortening has also been reported recently in relaxed muscle fibres on which length changes were externally imposed^{4,6,7}, and that system has allowed us to define more rigorously the circumstances in which stepwise shortening is observed. Here we report a high correlation between the frequency of 'steps' or 'pauses' and the translation velocity of the fibre past the measuring system, suggesting that stepwise shortening is not a physiological property of muscle but an instrumentation artefact.

To measure sarcomere length, we used two independent methods which have previously been used to study stepwise shortening: diffraction of laser light by the periodic arrangement of sarcomeres along the length of a muscle fibre⁸, and an optical imaging technique using a phase-locked loop (PLL)⁹. Changes of sarcomere length were brought about in single frog muscle fibres (see Fig. 1 legend) by anchoring one end and stretching and releasing the other. The effects of different translation velocities were studied either (1) by releasing at a constant speed but observing at different points along the fibre (exploiting the

gradient of velocity from the fixed to the moving end of a fibre), or (2) by varying the speed of the release and observing at a fixed point. In each case, natural markers on the fibres were used as points of reference. Both methods yield records showing the variation with time of sarcomere length at the point observed, from which the frequency of steps (evident in a variable proportion of experiments) could be calculated.

The results are summarized in Fig. 1 (laser diffraction) and Fig. 2 (optical imaging). In most experiments, the 'pauses' were inclined to the horizontal, giving the records a 'saw-tooth' appearance, but we have seen no justification for selecting only flat pauses in our analysis, as flat and inclined pauses often occurred on the same record. With each method, plots of frequency against the translation velocity of the point of the fibre observed fall on straight lines with correlation coefficients near unity. Extrapolation to zero shortening velocity predicts smooth shortening. The frequency of the oscillations we have observed appears to be determined only by the translation velocity of the fibre at the point observed, and not by the rate of change of sarcomere length. The latter is determined by the velocity of the moving end of the fibre, and was found to be very nearly constant along the length of the fibre, exhibiting only minor random variation.

The scatter in the data of Fig. 1 results, to a large extent, from errors in frequency estimation. With laser diffraction, steps appeared most often in areas of the fibre giving less than optimum signal quality and could not be found in most fibres. It was difficult to obtain cascades of clear steps throughout the length change. The PLL almost invariably produced regular cascades of steps, and frequency estimation was correspondingly accurate. Recent reports^{4,10} have also noted the greater abundance and regularity of steps with the PLL. Sarcomere length records were usually repeatable over multiple 'contractions' as in previous experiments^{1,3,11}.

In search of a mechanism which might generate these oscillations, the first-order diffraction line was monitored during sarcomere length changes. As the duration of a pause could be extended from <50 ms to >1 s simply by decreasing the length

Fig. 1 *a*, Step frequency plotted against translation velocity; results from laser diffraction⁸, beam diameter 100–200 μm . The lines were fitted by least-squares regression, $r=0.94$ – 0.98 . Frequency was measured by obtaining cascades of steps throughout the length change, and counting the number of cycles throughout. Translation was measured by following the movement of natural markers during the length change. Translation velocity was varied in two ways (see text). Method (1) was used on fibres of 2–4–83 and 4–4–83 and a combination of (1) and (2) on fibre of 5–4–83. *b*, Typical records from graph data. Upper records: translation velocity varied by studying two different points on the fibre, muscle release unchanged. The lower sarcomere length trace of each pair was obtained from the point nearer to the moving end of the fibre (note the high frequency of this record). Middle records: both the sarcomere length and the point along the fibre were changed; release velocity constant. Lower records: the position along the fibre, and the velocity and amplitude of length change varied from the same initial sarcomere length. All experiments were done on single anterior tibialis fibres of *Rana temporaria*, at 6–8 °C, in standard Ringer's solution. All fibres gave reproducible tetanic force records before and after the experiment. Fibres were viewed at all times through a $\times 40$ water immersion objective (Zeiss, n.a. 0.75).

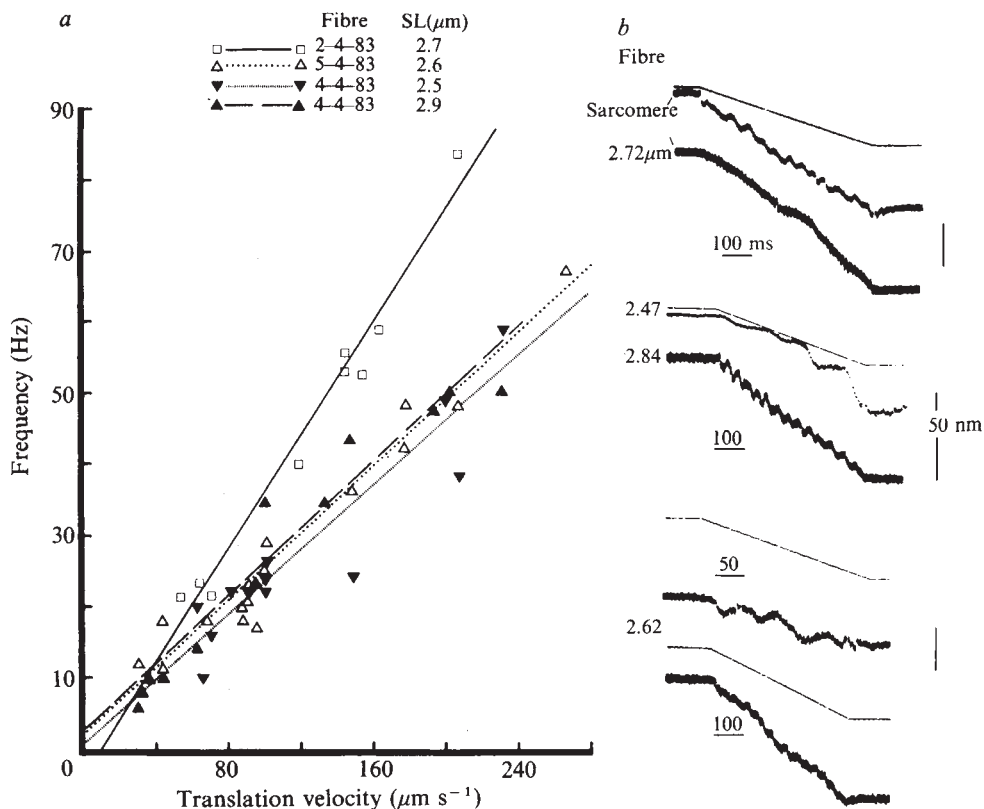


Fig. 2 *a*, Step frequency plotted against translation velocity. Inset shows continuation of data from fibre of 20-4-83. The lines were fitted by least-squares regression, $r = 1.0$ in both cases. Sarcomere length was determined by an on-line, direct imaging technique⁹, which uses a PLL to measure the spatial frequency of the microscopic image of the striation pattern, over 12–14 sarcomeres in series ($30\text{--}40 \times 5 \times 5 \mu\text{m}$). Representative records are shown in *b*; the translation velocity was varied by changing the velocity of release and stretch (fibre of 20-4-83). The data for 18-4-83 were derived from changing the velocity of release/stretch, and by moving the sensor along the fibre. The data points at 18, 22, 29, 32, 34 and 53 Hz were obtained by method (1) (see text), with all sarcomeres shortening at the same velocity.

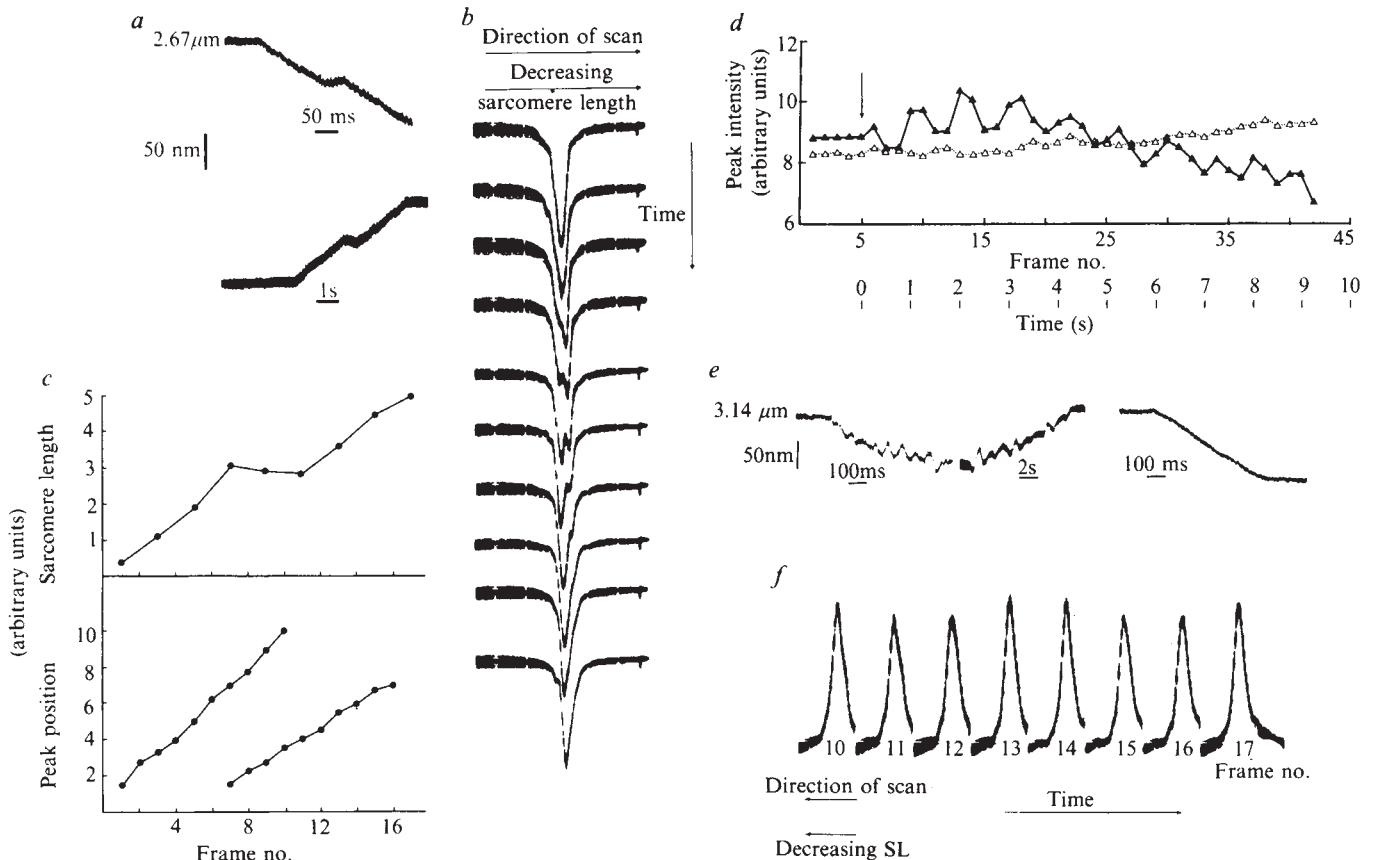
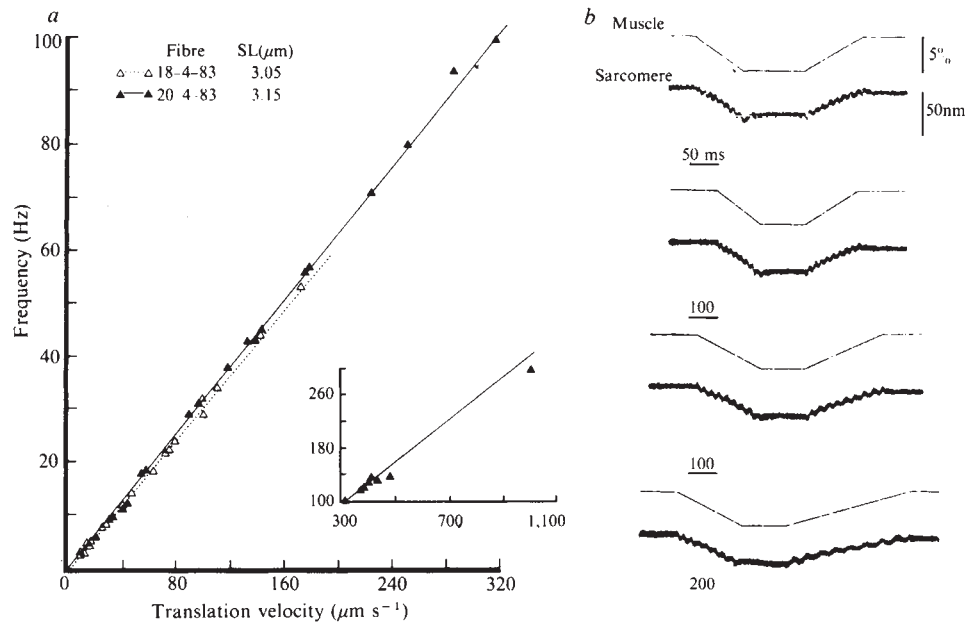


Fig. 3 *a*, A 50-ms pause, extended on the restretch to 1 s by slowing the length change. *b*, The first-order diffraction peak, as seen by the photodiode array, photographed at 4 frames per s during the extended pause. *c*, Sarcomere length, and the position of the two peaks of the first-order diffraction line (relative to an arbitrary reference line) plotted against time. Sarcomere length was plotted by electronically scanning the photodiode array from left to right (once every $256 \mu\text{s}$), integrating the light intensity, and computing the distance along the array at which 50% of the integral was reached. The 50% integral value from each scan was carried over to the next, and logic circuits were switched when the integral reached this value—the sarcomere length. Thus, each sarcomere length computation used the first-order light intensity value from the previous scan. It was assumed that the intensity does not change significantly from scan to scan. This assumption is valid for most purposes, but when used close to the resolution limit, as here, the errors may be significant. In this case, the appearance of a secondary peak increased the area under the curve at long sarcomere lengths, switching the logic prematurely, and resulting in an apparent halt in sarcomere lengthening, despite the fact that both peaks moved continuously, as shown in *c*. *d–f* Shows a second example in which the steps/oscillations are the result of periodic intensity changes in an otherwise smooth, near-symmetrical first-order diffraction line. In *d*, the peak intensity of the diffraction line is plotted against time during the slow restretch of the release/stretch sequence illustrated in the left-hand and centre records of *e*. Note how symmetrical the sarcomere length changes are, despite the difference in time base. The number of intensity change cycles equals the number of sarcomere length oscillations, and the earlier, larger sarcomere length changes on the stretch are associated with the greatest intensity changes. Consistent with the method of sarcomere length determination, an increase in intensity leads to an overestimate of sarcomere length. Frames 10–17 of the diffraction line are shown in *f*. For comparison the open triangles in *d* are from data taken during a typical smooth sarcomere length change (*e*, right-hand record).

change velocity, it was possible to photograph the diffraction line during a prolonged pause (Fig. 3a). Here the apparent pause can be accounted for by the formation of a secondary peak (Fig. 3b), leading to a mis-computation of sarcomere length, as the method is based on the assumption that there are no significant changes in peak intensity and symmetry between computations (see Fig. 3 legend). Small changes in first-order intensity alone may also produce steps (Fig. 3d-f). The analyses in Fig. 3 are representative of a total of 14, from three fibres, at sarcomere lengths of 2.5–3.9 μm . In all cases steps were associated with detectable changes in the diffraction line.

Thus, changes in intensity and the formation of secondary peaks in the first-order line are possible causes of steps/oscillations. Both phenomena might arise from Bragg diffraction^{2,12}, while myofibrillar sliding, resulting in periodic variation in myofibrillar skew, might also contribute to intensity fluctuations^{13,14}. Diffraction steps may therefore have several sources, explaining their often irregular appearance. The slope of the regression line for the PLL data indicates that one oscillatory cycle corresponds to the translation of one sarcomere past a given point in the optical field. This, and the regular appearance of PLL steps, suggests a single mechanism of generation, inherent in the method of sarcomere length determination.

Due to the rapid time course of a twitch, attempts to correlate step frequency with translation in active contractions would be difficult. However, one can estimate the upper and lower limits of translation velocity if fibre length and the extent and time course of internal shortening are known; all are readily available from our own and published data. From these calculations, translations of <20–70 μm in 50–150 ms might be expected; very similar values were calculated by Jacobson *et al.*¹⁵. From Figs 1 and 2, frequencies of <20–1,000 Hz are predicted, that is, half periods or pause durations of 1 to >25 ms. This range is consistent with that of published durations^{1,3–7,11,15}. The wide variety of step patterns observed in active contractions might be expected, because translation will be random relative to that occurring during the linear length changes imposed on resting fibres. Without studying active contractions, the above must remain speculative.

Many published properties of the stepwise shortening phenomenon can be explained on the basis of a translation-dependent oscillation. The increase in pause duration observed under: increased load¹¹, decreased sarcomere length (and concomitant decrease in shortening velocity)¹¹ and near the peak of a twitch^{3,11}, can all be accounted for by the decrease in internal movements in these conditions. Decreasing translation velocity would decrease the oscillation frequency and hence increase pause duration.

That step generation may result from changes in the diffraction pattern is consistent with the observation that steps are more common in cardiac than in skeletal preparations^{1,3}; it is well known that cardiac muscle gives poorer diffraction patterns.

Our studies revealed other features suggesting a non-physiological origin for steps. A 20 °C change in temperature had no effect on steps during passive length changes. The occurrence of steps at sarcomere lengths around 4.0 μm , indistinguishable from those at 2.4 μm (ref. 7), clearly indicates that steps do not result from actomyosin interaction, unless there is a substantial change in filament length during the passive stretch of sarcomeres beyond 3.65 μm .

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- Pollack, G. H., Iwazumi, T., ter Keurs, H. E. D. J. & Shibata, E. F. *Nature* **268**, 757–759 (1977).
- Rüdel, R. & Zite-Ferency, F. *Nature* **278**, 573–575 (1979).
- Pollack, G. H., Vassallo, D. V., Jacobson, R. C., Iwazumi, T. & Delay, M. J. in *Cross-bridge Mechanism in Muscle Contraction* (eds Sugi, H. & Pollack, G. H.) 23–40 (University of Tokyo Press, 1979).

- Pollack, G. H. *et al.* in *Contractile Mechanisms in Muscle* (eds Pollack, G. H. & Sugi, H.) (Plenum, New York, in the press).
- Delay, M. J., Ishide, N., Jacobson, R. C., Pollack, G. H. & Tirosh, R. *Science* **213**, 1523–1525 (1981).
- Lacktis, J. W., Brozovich, F. V. & Pollack, G. H. *Biophys. J.* **37**, 358a (1982).
- Lacktis, J. W., Brozovich, F. V. & Pollack, G. H. *Biophys. J.* **41**, 264a (1983).
- Iwazumi, T. & Pollack, G. H. *IEEE Trans. Biomed. Eng.* **26**, 86–93 (1979).
- Myers, J., Tirosh, R., Jacobson, R. C. & Pollack, G. H. *IEEE Trans. Biomed. Engng* **29**, 463–466 (1982).
- Jacobson, R. C., Tirosh, R., Delay, M. J. & Pollack, G. H. *Biophys. J.* **33**, 223a (1981).
- Vassallo, D. V. & Pollack, G. H. *Circulation Res.* **51**, 37–42 (1982).
- Rüdel, R. & Zite-Ferency, F. *J. Physiol., Lond.* **290**, 317–330 (1979).
- Oba, T., Baskin, R. J. & Lieber, R. L. *J. Muscle Res. Cell Motility* **2**, 215–218 (1981).
- Yeh, Y., Baskin, R. J., Lieber, R. L. & Roos, K. P. *Biophys. J.* **29**, 519–522 (1980).
- Jacobson, R. C., Tirosh, R., Delay, M. J. & Pollack, G. H. *J. Muscle Res. Cell Motility* (in the press).

● A REPLY from Dr H. E. Pollack has been received and is being considered.

Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton

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The cytoskeleton underlying the membrane of erythrocytes is thought to control changes in cell shape such as the diskocyte to the echinocyte. Since the binding of lectins to the transmembrane protein glycophorin blocks the cell shape change, we have proposed that the cytoplasmic end of glycophorin is linked to the cytoskeleton¹. Here we show that the cytoskeletal protein known as band 4.1 specifically associates with the cytoplasmic domain of glycophorin on inside-out erythrocyte membrane vesicles and also with glycophorin reconstituted into phosphatidylcholine vesicles. The binding of band 4.1 to glycophorin is saturable and inhibitable by antibodies which bind specifically to the cytoplasmic domain of glycophorin. We therefore believe that band 4.1 protein provides the link between glycophorin and the cytoskeleton.

Glycophorin was isolated^{2,3}, desialated and incorporated into phosphatidylcholine vesicles (dGP-vesicles) by dialysis of a mixed micellar solution of octyl glucoside, phosphatidylcholine and desialoglycophorin⁴. The resulting dGP-vesicles are large enough to be easily sedimented using the ultracentrifuge. It is this property which allows proteins that associate with the vesicles to be separated from proteins that remain free in solution.

Membrane skeletal proteins were sequentially solubilized from isolated erythrocyte membranes⁵ by extracting with buffered solutions of various ionic strengths^{6,7}. The membrane skeletal extracts are enriched in specific proteins (refs 7, 8 and Fig. 1). The extracted proteins were combined with dGP-vesicles and incubated at 37 °C. The dGP-vesicles with bound proteins were then sedimented in the ultracentrifuge and the supernatant was removed. Protein from the vesicle pellet was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 1). As a control an equivalent amount of phosphatidylcholine vesicles (PC-vesicles) prepared identically to dGP-vesicles but without glycophorin were combined with membrane skeletal proteins and analysed in an identical fashion (Fig. 1). The SDS-PAGE shows that only one membrane skeletal protein (band 4.1) specifically bound to the dGP-vesicles. Although several proteins bound nonspecifically to both kinds of vesicles, only band 4.1 showed a large relative increase in the amount bound to dGP-vesicles. Quantification of the amount of band 4.1 bound⁸ shows that dGP-vesicles bound 16-fold more band 4.1 (4.7 μg) than did PC-vesicles (0.3 μg) (Fig. 1, lanes f, g).

In a similar experiment, membrane skeletal proteins extracted with 1 M KCl (mainly spectrin, bands 2.1, 6, 4.9 and 4.1) were

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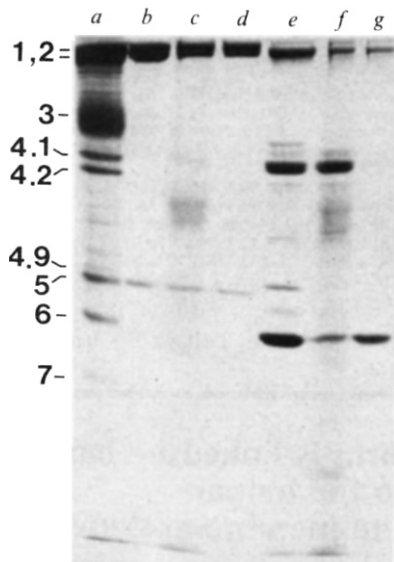


Fig. 1 SDS-PAGE of membrane skeletal protein extracts and membrane skeletal proteins bound to dGP- and PC-vesicles. Samples are: *a*, membrane proteins, 25 μ g; *b*, low ionic strength extract, 7 μ g protein; *c*, dGP-vesicles + low ionic strength extracted protein; *d*, PC-vesicles + low ionic strength extracted protein; *e*, 1 M KCl extracted protein, 14 μ g protein; *f*, dGP-vesicles + 1 M KCl extracted protein; *g*, PC-vesicles + 1 M KCl extracted protein.

Methods: Isolated glycophorin² (15 mg ml⁻¹) was desialated with *Clostridium perfringens* neuraminidase (0.1 U ml⁻¹) at pH 5.0 for 1 h at 37 °C. The desialoglycophorin was then reconstituted into large phosphatidylcholine vesicles⁴. To a thin film of phosphatidylcholine was added octyl- β -D-glucopyranoside (octyl glucoside) and desialoglycophorin in water. The resulting mixed micellar solution of phosphatidylcholine (36 mM), octyl glucoside (340 mM) and desialoglycophorin (0.12 mM) was extensively dialysed against 140 mM NaCl, 5 mM sodium phosphate, 3.0 mM NaN₃, 0.5 mM DL-dithiothreitol (DTT) at pH 7.6. The solution of dGP-vesicles was applied to a 2.0 $\times$ 38 cm Sepharose CL-4B column equilibrated with 130 mM KCl, 10 mM NaCl, 5 mM sodium phosphate, 3 mM NaN₃, 1 mM EDTA, 0.5 DTT at pH 7.6 (130 mM KCl buffered). Vesicles with reconstituted desialoglycophorin eluted in the column void. Control vesicles (PC-vesicles) were prepared identically, except without desialoglycophorin. Membrane skeletal proteins were extracted from isolated erythrocyte membranes using: (1) low ionic strength buffer to extract spectrin and actin and (2) 1 M KCl buffer to extract primarily bands 2.1, 4.1, 6 and residual spectrin (refs 5 and 6 describe the complete buffers). The cytoskeletal protein solutions were dialysed against 130 mM KCl, 10 mM NaCl, 5 mM sodium phosphate, 0.5 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and then combined with dGP- and PC-vesicles. The final concentration of membrane skeletal protein was 20 μ g ml⁻¹; glycophorin concentration was 50 μ g ml⁻¹. The concentration of phosphatidylcholine was 250 μ g ml⁻¹ for both dGP- and PC-vesicles. Solutions of membrane skeletal proteins and vesicles were incubated at 37 °C for 20 min then centrifuged at 150,000g for 1 h at 2 °C. The vesicle pellets were solubilized in sample buffer, applied to a SDS-PAGE and developed according to Laemmli's method²².

combined with either dGP-vesicles or PC-vesicles. Following incubation at 37 °C the vesicle solutions were applied to a Sepharose 4B-CL column. Both kinds of vesicles eluted from the column in the void and were completely separated from proteins which were not bound (Fig. 2A). The vesicles were then analysed by SDS-PAGE as above, comparing the proteins remaining bound to dGP-vesicles (Fig. 2A, lane *a*) with proteins binding to PC-vesicles (lane *b*). In these conditions dGP-vesicles retained about 40-fold more band 4.1 than did PC-vesicles: 3.9 μ g compared with <0.1 μ g of bound band 4.1, respectively. Spectrin was the only other protein which bound to the vesicles. The dGP-vesicles also retained more spectrin than did PC-vesicles, possibly due to the spectrin-band 4.1 association^{6,7}.

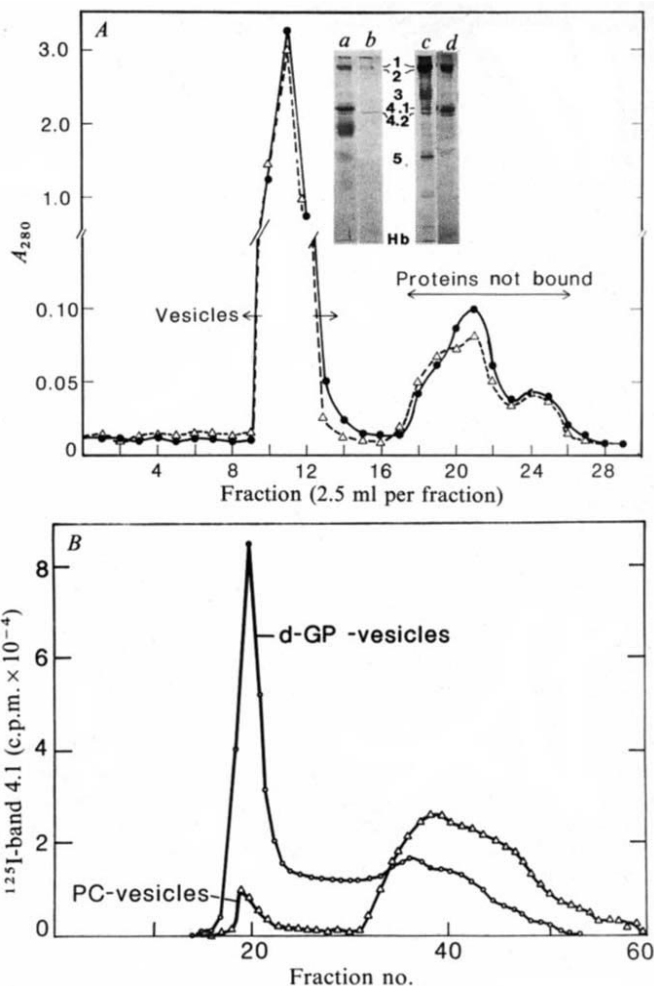


Fig. 2 *A*, Band 4.1 remains bound to dGP-vesicles (Δ) after chromatography of Sepharose CL-4B, but not to PC-vesicles ($\bullet$). The samples on SDS-PAGE are: *a*, proteins bound to dGP-vesicles; *b*, proteins bound to PC-vesicles; *c*, 20 μ g of total membrane proteins; *d*, 12 μ g of protein extracted from membranes with 1 M KCl buffer. *B*, Elution profile of ¹²⁵I-protein 4.1 which was combined with dGP-vesicles ($\circ$) or with PC-vesicles (Δ).

Methods: *A*, The 1 M KCl extracted membrane skeletal proteins (100 μ g ml⁻¹) were combined with dGP-vesicles (100 μ g ml⁻¹ glycophorin) or PC-vesicles. The solutions, after incubation at 37 °C for 20 min, were applied to a 2 $\times$ 38 cm Sepharose CL-4B column. Vesicles eluted from the column in the void together with bound protein. The vesicles which eluted from the column were concentrated by centrifugation (as in Fig. 1) and the protein in the vesicle pellet was analysed by SDS-PAGE. *B*, Purified band 4.1 was labelled with ¹²⁵I Bolton-Hunter reagent^{6,7}. The ¹²⁵I-labelled band 4.1 (50 μ g ml⁻¹) was combined with dGP-vesicles (200 μ g ml⁻¹ glycophorin) or an equivalent amount of PC-vesicles; after incubation the solution was applied to a 2 $\times$ 38 cm Sepharose CL-4B column as above. The elution profile was monitored by following the ¹²⁵I label. The vesicle peak was determined by turbidity measurements at 280 nm.

To establish that band 4.1 associates specifically with dGP-vesicles without participation of other proteins, band 4.1 was purified and labelled with ¹²⁵I using the Bolton-Hunter reagent^{7,8}. Purified band 4.1 showed the characteristic major doublet on Laemmli SDS-PAGE, with two minor bands⁹, above and below band 4.1, representing about 15% of the total applied proteins. All three bands are immunologically cross-reactive with anti-band 4.1 antibody⁹. The ¹²⁵I-labelled band 4.1 was combined with dGP-vesicles or PC-vesicles and the vesicle solutions were applied to a Sepharose 4B column. Elution of band 4.1 was monitored by following the ¹²⁵I label. The elution profile of band 4.1 from the Sepharose column (Fig. 2B) demonstrates that a large fraction (37%) of the ¹²⁵I-band 4.1 remained bound

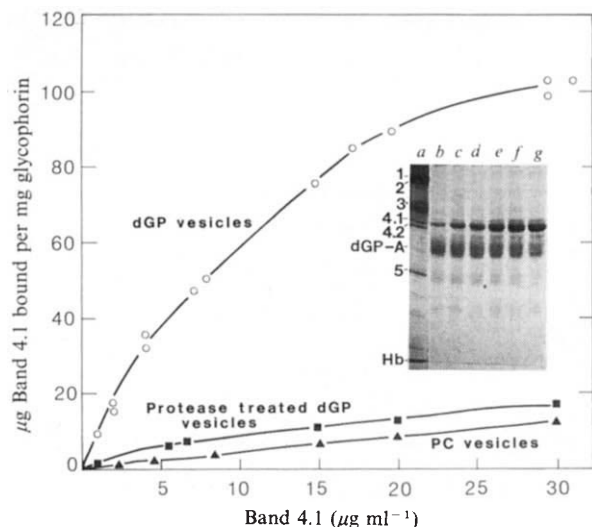


Fig. 3 Binding isotherms of native protein 4.1 to dGP-vesicles (○), protease-treated dGP-vesicles (■) and PC-vesicles (▲). An SDS-PAGE of band 4.1 bound to dGP-vesicles is shown. Samples are: *a*, 30 μg of total membrane protein; *b*–*g*, protein from the dGP-vesicle pellet, the band 4.1 concentration being varied over 2–30 μg ml⁻¹.

Methods: The binding isotherms were determined by combining dGP-vesicles (60 μg ml⁻¹ glycoporphin) with increasing amounts of native band 4.1. The solutions were incubated as described in Fig. 2 legend, followed by centrifugation of the dGP-vesicles with bound protein. The resulting vesicle pellet was prepared for SDS-PAGE as in Fig. 1. To the SDS-PAGE (separate lanes) was applied between 1 and 10 μg per lane of ultra-pure bovine serum albumin (BSA) as a protein standard. The amount of band 4.1 in each lane of the SDS-PAGE was quantitated by extracting and quantitating the Coomassie blue stain. The amount of dye bound was compared with a standard curve generated with known amounts of BSA applied to the same SDS-PAGE⁸. From the standard curve the amount of band 4.1 bound was determined as a ratio of μg band 4.1 bound per mg glycoporphin. The SDS-PAGE shows that the desialoglycophorins all migrate well below band 4.1 on the SDS-PAGE and thus do not interfere with dye extraction from the 4.1 band; ~70 μg per lane glycoporphin was applied. Binding is plotted in terms of μg band 4.1 bound per mg reconstituted glycoporphin versus the total band 4.1 concentration in μg ml⁻¹ (○). Nonspecific binding of band 4.1 was determined using an equivalent amount of phosphatidylcholine vesicles without incorporated desialoglycophorin (▲). Desialoglycophorin reconstituted into phosphatidylcholine vesicles was cleaved with a 20:1 weight ratio of desialoglycophorin to both trypsin and chymotrypsin at 37°C for 4 h. Protease activity was destroyed by addition of 0.1 mM diisopropyl fluorophosphate (DFP). Binding of band 4.1 to protease-treated dGP-vesicles is plotted in terms of μg band 4.1 bound per mg of original glycoporphin (■).

and co-eluted with the dGP-vesicles. In contrast, only a small fraction (2.5%) of the ¹²⁵I label co-eluted with PC-vesicles. Analysis of these vesicles by SDS-PAGE showed that 85–90% of the ¹²⁵I label bound to dGP-vesicles migrated with band 4.1. The majority (70–80%) of the ¹²⁵I label bound to PC-vesicles migrated with the small molecular weight minor protein band on SDS-PAGE, not with band 4.1. The dGP-vesicles in these conditions retained 60-fold more band 4.1 than did an equivalent amount of PC-vesicles.

To demonstrate further that band 4.1 associates specifically with reconstituted glycoporphin, a binding isotherm of band 4.1 to dGP-vesicles was determined. The binding isotherm (Fig. 3), determined using native band 4.1, illustrates that the association of 4.1 with dGP-vesicles is saturated at ~110 μg of band 4.1 bound per mg glycoporphin. The binding constant is $1.1 \pm 0.4 \times 10^7 \text{ M}^{-1}$. Nonspecific binding was determined using an equivalent amount of PC-vesicles (Fig. 3). The band 4.1 binding sites on dGP-vesicles are removed by proteolysis with trypsin and chymotrypsin, indicating that band 4.1 binding requires a protease-sensitive peptide (Fig. 3).

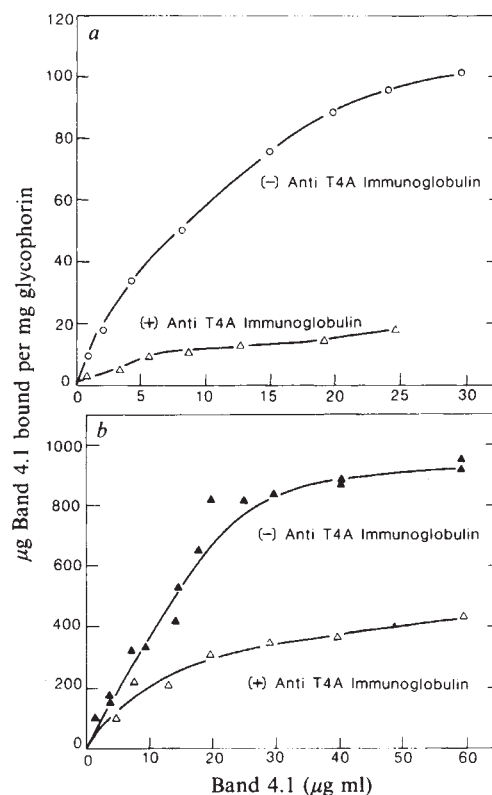


Fig. 4 *a*, Binding isotherm of ¹²⁵I-Bolton-Hunter labelled band 4.1 to dGP-vesicles (○) and inhibition of ¹²⁵I-band 4.1 binding to dGP-vesicles by anti-glycophorin cytoplasmic domain antibody (anti-T4A antibody) (Δ). *b*, Binding of ¹²⁵I-band 4.1 to IOVs (▲) and inhibition of ¹²⁵I-band 4.1 binding to IOVs using anti-T4A antibody (Δ).

Methods: *a*, Band 4.1 (200 μg) was labelled with 0.2 mCi of ¹²⁵I-Bolton-Hunter reagent⁷. Labelled band 4.1 (0.8 mCi mg⁻¹) with full biological activity was isolated by binding ¹²⁵I-band 4.1 to dGP-vesicles, separating the biologically active ¹²⁵I-band 4.1 from inactive ¹²⁵I-band 4.1 by centrifugation or chromatography as in Fig. 2*B* and then eluting the bound ¹²⁵I-band 4.1 from the dGP-vesicles with 1 M KCl buffer. The ¹²⁵I-band 4.1 was dialysed against 130 mM KCl buffer and diluted with cold band 4.1 to a specific activity of ~1 μCi per mg. This ¹²⁵I-band 4.1 was used for band 4.1 binding to dGP-vesicles. (Sheep antiserum raised against the cytoplasmic domain of glycoporphin A¹¹ (cyanogen bromide fragment 2) was a gift from Drs Marchesi and Furthmayr, Yale University.) Pure antibody was isolated by affinity chromatography on the T4A(B) tryptic fragment of glycoporphin A which was coupled to Sepharose-4B with cyanogen bromide²³. To inhibit band 4.1 binding to dGP-vesicles, the vesicles were pretreated with antibody at a 1:1 antibody/glycophorin molar ratio. However, as glycoporphin is asymmetrically incorporated into phospholipid vesicles it is likely that at most only 20% of the glycoporphins have available cytoplasmic domains⁴, thus a 5:1 molar ratio is more likely. At such a molar ratio of antibody to glycoporphin cytoplasmic domain the antibody binding sites are likely to be saturated. *b*, IOVs were prepared according to refs 14 and 15. The amount of glycoporphin in these IOVs was determined by assaying for sialic acid²⁴, assuming 70% of total sialic acid is attached to glycoporphins and that glycoporphins are 30% sialic acid by weight. The concentration of glycoporphin in IOVs used for binding was 4 μg ml⁻¹ (30 μg ml⁻¹ total protein). The amount of bound ¹²⁵I-band 4.1 was determined by counting the vesicle pellet. Inhibition of band 4.1 binding to IOVs was accomplished by using a fivefold antibody excess as above.

Reconstitution of glycoporphin into phosphatidylcholine vesicles results in an asymmetric orientation of glycoporphin across the lipid bilayer. The majority of the carbohydrate-bearing domains are located on the outside surface of the vesicles^{4,10}. Such asymmetric orientation corresponds well with the binding isotherm, as asymmetric orientation of glycoporphin would result in the low band 4.1:glycophorin stoichiometry that is observed

at saturation, if band 4.1 associates with the cytoplasmic domain of glyophorin.

Further evidence that band 4.1 associates with the cytoplasmic domain of glyophorin is provided by antibodies directed against the cytoplasmic domain of glyophorin A. These antibodies were used to inhibit band 4.1 binding to vesicles containing glyophorin. Antiserum was raised in sheep immunized against the cyanogen bromide fragment (CB2) containing the cytoplasmic domain of glyophorin A (ref. 11). Pure antibody specific for the cytoplasmic domain of glyophorin was isolated by affinity chromatography using the T4A fragment of glyophorin A^{11,12} as the affinity ligand attached to Sepharose 4B. The T4A fragment is the major portion of the glyophorin A cytoplasmic domain^{11,12}.

Band 4.1 binding and inhibition of binding was monitored with ¹²⁵I-labelled band 4.1 (refs 7, 8). The binding isotherm of ¹²⁵I-band 4.1, with full biological activity towards dGP-vesicles (Fig. 4a), compares well with that determined using native band 4.1 (Fig. 3). Anti-T4A antibody, preincubated with dGP-vesicles before addition of protein 4.1, blocked 70–80% of the band 4.1 binding sites on dGP-vesicles. However, when pure glyophorin A was reconstituted into phosphatidylcholine vesicles, band 4.1 specifically bound to these vesicles and anti-T4A antibody completely inhibited (>90%) the band 4.1 binding. Moreover, when glyophorin B was reconstituted into phosphatidylcholine vesicles, band 4.1 specifically bound to these vesicles, but the binding was not inhibited by anti-T4A antibody (unpublished results). Such behaviour is expected, as anti-T4A antibody does not cross-react with glyophorin B¹³.

Inverted erythrocyte membrane vesicles (IOVs) were isolated from fresh erythrocyte membranes⁵ and stripped of membrane skeletal proteins^{14,15}. The stripped IOVs were used to establish a binding isotherm of ¹²⁵I-labelled band 4.1 to the cytoplasmic surface of the erythrocyte membrane (Fig. 4b). The binding isotherm shows saturation and approximately the same binding affinity as band 4.1 binding to dGP-vesicles. At saturation 880 µg of band 4.1 are bound per mg of glyophorin. Pretreatment of stripped IOVs with anti-T4A antibody blocked at least 60% of the band 4.1 binding sites (Fig. 4b). Only the cytoplasmic domain of glyophorin A was blocked by anti-T4A antibody. Thus, at least 60% of the band 4.1 binding sites are contributed by glyophorin A. The remaining binding sites may be contributed by the cytoplasmic domains of glyophorin B and glyophorin C which would still be available for binding¹⁶. Thus in En(-a) erythrocytes, which are thought to be devoid of glyophorin A¹⁷, glyophorins B and C could still contribute enough membrane attachment sites to accommodate all of the protein 4.1 molecules in the cell.

The above results provide evidence that band 4.1 specifically associates with the cytoplasmic domain of glyophorin A, and also possibly glyophorin B. Previously, band 4.1 was shown to associate tightly with spectrin^{6,7}. The binding site for band 4.1 on spectrin is located in close proximity to the spectrin-actin association site^{6,7}. Furthermore, association of band 4.1 with spectrin markedly enhanced the interaction between spectrin and actin protofilaments^{18–21}. As such, the effect of band 4.1 on the spectrin-actin association could have a regulatory role in controlling the dynamics of membrane skeleton change. Control of membrane skeletal change may in turn be mediated by externally bound lectins via the interaction between transmembrane glyophorin and band 4.1.

8. Fenner, C., Traut, R. R., Mason, D. T. & Wikman-Coffel, J. *Analyt. Biochem.* **63**, 595–602 (1975).
9. Cohen, C. M., Foley, S. F. & Korsgren, C. *Nature* **299**, 648–650 (1982).
10. Ong, R. L., Marchesi, V. T. & Prestegard, J. H. *Biochemistry* **20**, 4283–4292 (1981).
11. Cotmore, S. F., Furthmayr, H. & Marchesi, V. T. *J. molec. Biol.* **113**, 539–553 (1977).
12. Tomita, M., Furthmayr, H. & Marchesi, V. T. *Biochemistry* **17**, 4756–4770 (1978).
13. Furthmayr, H. *Nature* **271**, 519–524 (1978).
14. Hargreaves, W. R., Giedd, K. N., Verkleij, A. & Branton, D. *J. biol. Chem.* **255**, 11965–11972 (1980).
15. Steck, T. L., Weinstein, R. S., Strauss, J. H. & Wallach, D. F. H. *Science* **168**, 255–257 (1970).
16. Mueller, T. J. & Morrison, M. in *Erythrocyte Membranes* Vol. 2, 95–112 (Liss, New York, 1981).
17. Dahr, W., Uhlenbruck, G., Wagstaff, W. & Leikola, J. J. *J. Immunogenet.* **3**, 383–393 (1976).
18. Ungewickell, E., Bennett, P. M., Calvert, R., Ohanian, V. & Gratzner, W. G. *Nature* **280**, 811–814 (1979).
19. Fowler, V. & Taylor, D. L. *J. Cell Biol.* **85**, 361–376 (1980).
20. Brenner, S. L. & Dorn, E. D. *J. biol. Chem.* **254**, 8620–8627 (1979).
21. Cohen, C. M. & Korsgren, C. *Biochem. biophys. Res. Commun.* **97**, 1429–1435 (1980).
22. Laemmli, V. *Nature* **227**, 680–681 (1970).
23. March, S. C., Parikh, I. & Cuatrecasas, P. *Analyt. Biochem.* **60**, 149–152 (1974).
24. Warren, L. *J. biol. Chem.* **234**, 1971–1975 (1959).

Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas

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An important feature of the development of many human and animal tumours is the appearance of pre-malignant benign lesions, some of which undergo further changes during progression to malignancy^{1–4}. Many of the currently accepted concepts of multi-stage carcinogenesis have been developed using an experimental model based on the chemical induction of tumours in mouse skin^{3,4}. In this system, many of the pre-malignant papillomas which arise are promoter-dependent, and appear to regress if promoter treatment is interrupted, whereas others progress to form autonomous benign lesions and, in some cases, malignant carcinomas⁵. Although the number and nature of the events leading to malignancy are not known, DNA transfection experiments have led to the identification of several genes which may be qualitatively altered in tumour cells (see ref. 6 for review). We have previously shown that DNA from transplantable mouse skin carcinomas induced by chemical carcinogens has the ability to transform NIH/3T3 cells, and that the gene responsible for the transformation is an activated form of the mouse cellular Harvey-ras gene (*c-ras*^H)⁷. We have now investigated the stage of carcinogenesis at which the proto-oncogene acquires transforming activity. We demonstrate that primary papillomas induced by chemical carcinogens in two different mouse strains have an activated *c-ras*^H gene. This constitutes the first report of a benign tumour which contains DNA with detectable transforming activity. In addition, steady-state levels of *c-ras*^H gene transcripts are elevated in the papillomas as compared with normal epidermis.

Both benign and malignant skin lesions can be induced in mouse skin by sequential treatment with initiators and promoters of carcinogenesis^{3,4}. Tumours were induced in the dorsal skin of Sencar mice, a strain bred for sensitivity to chemical carcinogens^{8,9}, by initiation with dimethylbenzanthracene (DMBA) followed by multiple treatments with the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). In this strain individual papillomas and carcinomas are large enough to provide sufficient DNA for analysis by transfection into NIH/3T3 cells. Table 1 shows the results obtained when several primary tumours were tested for transforming activity. Of five DNA samples prepared from papillomas, four showed positive results in transfection assays. Similarly, two out of three carcinomas induced foci of transformed cells in NIH-3T3 fibroblasts. In the same series of experiments, DNA from normal

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1. Anderson, R. A. & Lovrien, R. J. *Cell Biol.* **85**, 534–548 (1980); *Nature* **292**, 158–161 (1981).
2. Marchesi, V. T. & Andrews, E. P. *Science* **174**, 1247–1248 (1971).
3. Furthmayr, H., Tomita, M. & Marchesi, V. T. *Biochem. Biophys. Res. Commun.* **65**, 113–121 (1975).
4. Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C. & Reynolds, J. A. *Biochemistry* **20**, 833–840 (1981).
5. Dodge, J. T., Mitchell, C. & Hanahan, D. J. *Archs Biochem. Biophys.* **100**, 119–130 (1963).
6. Tyler, J. M., Hargreaves, W. R. & Branton, D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5192–5196 (1979).
7. Tyler, J. M., Reinhardt, B. N. & Branton, D. *J. biol. Chem.* **255**, 7034–7039 (1980).

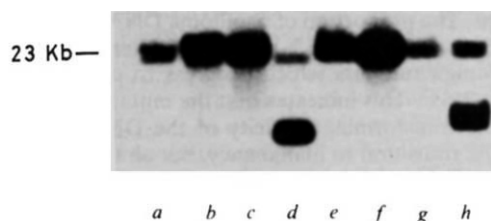


Fig. 1 Southern blot analysis of sequences related to the *v-ras^H* gene in NIH/3T3 transformants induced by primary mouse papilloma and carcinoma DNAs. The DNAs were from the following sources: *a*, NIH/3T3; *b*, NIH/3T3 transformant obtained by transfection with primary carcinoma DNA; *c-f*, *h*, transformants induced by different papilloma DNAs; *g*, spontaneously arising transformant.

Methods: Tumours were induced on the dorsal skins of Sencar or NMRI mice by sequential treatment with DMBA and TPA^{3,4}. High molecular weight DNA was isolated as previously described⁷. DNA (20 µg) from NIH/3T3 transformants was digested with *EcoRI*, electrophoresed through a 1% agarose gel, blotted onto nitrocellulose and hybridized with a nick-translated *v-ras^H* specific probe (BS9)²². Blots were washed to a final stringency of 0.5×SSC at 65 °C and exposed to Kodak XAR5 film with intensifying screens at -70 °C.

epidermis or from epidermis induced to proliferate by a single treatment with TPA failed to induce any stable transformants. Preliminary experiments using NIH mice (A.B. and J.S., unpublished results) also suggest that chronic treatment with TPA (three times weekly for 7 weeks) does not cause activation of transforming genes to a level detectable by the NIH/3T3 transfection assay. When primary transformants induced either by papilloma or carcinoma DNAs were grown in bulk culture, all the high molecular weight DNAs from these cells were positive when tested in second rounds of transfection into NIH/3T3 fibroblasts, indicating that a stable transforming gene was being transferred from both papillomas and carcinomas.

It has previously been noted that some papillomas can develop on the dorsal skin of Sencar mice after repeated promoter treatment but without any prior initiation⁹. This suggested that these mice may have a constitutive hereditary mutation which predisposes to the development of skin tumours. However, the lack of transforming ability of DNA from normal Sencar epidermis or from proliferating epidermis treated with TPA suggests that the transforming activity of the papilloma DNA cannot be attributed to a constitutive mutation in this mouse strain.

It remained possible, however, that Sencar papillomas might be abnormally 'advanced' in terms of tumour progression, and that transforming activity in other less sensitive mouse strains might only be acquired at the carcinoma stage. For this reason, papillomas were induced in NMRI mice and tested for transforming activity. Two independent DNA preparations from pooled small papillomas were positive in transfection assays, whereas liver or brain DNAs from the same inbred mouse strain were negative.

The transformation efficiencies with all of the primary tumour DNAs, including both papillomas and carcinomas, were about 5–10-fold lower than DNAs from the human EJ bladder carcinoma cell line^{10–13} or a transplanted mouse carcinoma⁷. Similar low efficiencies have also been reported for a number of primary human tumours¹⁴. Note that the transforming efficiencies of all papilloma DNA samples were at least as high as that of the primary carcinomas, indicating that the results cannot be attributed to the presence of a small, histologically undetectable proportion of malignant carcinoma cells within some of the papillomas. In addition, the similar efficiencies obtained with the pooled small NMRI papillomas and the individual large Sencar papillomas demonstrate that papilloma size *per se* is not an important determinant of transforming gene concentration.

We have previously shown that transplanted carcinomas of NMRI mice contain an activated form of the *c-ras^H* gene⁷. To determine whether the *ras^H* gene is reproducibly activated during chemical carcinogenesis in the mouse skin model system, DNA from transformants induced by papilloma or carcinoma

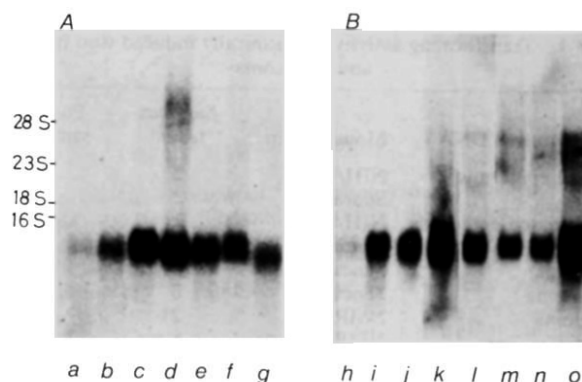


Fig. 2 A, Northern blot of *c-ras^H* transcripts in primary tumours. Total cellular RNAs were isolated from the following sources: *a*, normal epidermis; *b*, TPA-treated epidermis; *c-e*, individual papillomas from Sencar mice; *f*, pooled small papillomas from NMRI mice; *g*, Sencar carcinoma. B, Transcripts of *c-ras^H* sequences in NIH/3T3 transformants. Sources of the RNA were: *h*, NIH/3T3 cells; *i, j*, NIH/3T3 transformants induced by transplanted NMRI carcinoma DNA⁷; *k-n*, transformants induced by papilloma DNA; *o*, transformant induced by primary carcinoma DNA.

Methods: Frozen tumours or epidermis were ground in liquid nitrogen and lysed in 5 M guanidinium thiocyanate, 50 mM Tris-HCl (pH 7.0), 50 mM EDTA and 5% (v/v) 2-mercaptoethanol. Total cellular RNA was isolated from the lysate by centrifugation through a 5.7 M CsCl, 50 mM EDTA (pH 7.0) cushion, followed by ethanol precipitation. Aliquots (10 µg) of RNA were heated to 60 °C for 5 min in 50% deionized formamide, 2.2 M formaldehyde, 20 mM MOPS buffer, cooled rapidly, electrophoresed through 1% agarose gels in MOPS buffer (20 mM morpholinopropanesulphonic acid, 5 mM Na acetate, 1 mM EDTA pH 7.0), blotted onto nitrocellulose and hybridized to the ³²P-labelled *v-ras^H*-specific probe.

DNAs was isolated and analysed by Southern blotting and hybridization with a *ras^H*-specific probe. Newly acquired *ras^H* genes in transformed foci can be detected either by comparing band intensities with appropriate controls on genomic Southern blots or, more convincingly, by the presence of additional restriction fragments containing the extra gene(s) due to loss of a restriction site on integration. Figure 1 shows that DNAs from all of the foci induced by both papillomas and carcinomas exhibit a large increase in the intensity of hybridization with a *ras^H* probe, compared with normal 3T3 controls. In addition, some of the foci (lanes *d, h*) have an additional restriction fragment containing the extra *ras^H* genes, with the NIH/3T3 gene visible at the normal 23-kilobase (kb) position. No evidence for increased intensity of hybridization or additional restriction fragments were observed in spontaneously arising foci isolated from the same experiments (lane *g*). The amplification of the *ras^H* gene in the foci is not attributable to transfer of multiple copies from the original tumour cells, as these appear to contain only single copy *ras^H* sequences (data not shown). On probing the DNA of the foci with a Kirsten-*ras* specific probe, the intensity and pattern of hybridization in all foci was virtually identical to that in the 3T3 controls (data not shown), suggesting that the transfer and amplification had probably only occurred with the *ras^H* gene.

To determine whether *c-ras^H* transcripts are elevated in mouse skin tumours, RNA was prepared from a series of primary tumours and hybridized on Northern blots with a *ras^H*-specific probe. Figure 2A shows that the concentration of the major 1.4-kb transcript is increased in a series of papillomas relative to normal epidermis. This increase is apparent in individual Sencar papillomas (lanes *c-e*), in pooled small papillomas from NMRI mice (lane *f*), and, to a lesser extent, in a Sencar primary carcinoma (lane *g*). In view of the difficulties involved in estimating RNA concentrations by Northern blot hybridizations with a single probe, the blot shown in Fig. 2A has been re-hybridized with a cloned probe which is complementary to the ubiquitous, highly abundant cytoplasmic 7S RNA¹⁵. This demonstrated that

Table 1 Transforming activity of chemically induced skin papillomas and carcinomas

Source of DNA	Mouse strain	Samples tested	Positive samples‡
Normal epidermis	NIH/Swiss	2	0
	Sencar	2	0
TPA-treated epidermis	NIH/Swiss	1	0
	Sencar	1	0
Papilloma	Sencar	5*	4*
Carcinoma	Sencar	3	2
Papilloma	NMRI	2†	2†
Liver	NMRI	1	0
Brain	NMRI	1	0

Frozen tissues were ground in liquid nitrogen and DNA samples were prepared by resuspension in guanidinium thiocyanate and spinning through CsCl as previously described⁷. Transfections were carried out by adding 20 µg high molecular weight DNA to 25 cm² flasks seeded 24 h earlier with 3×10^5 cells per flask. Cells were maintained and foci scored as described earlier⁷. Epidermal cells were prepared from groups of 10–20 mice by scraping with a rounded scalpel after heating the skins in water at 55 °C for 30 s. Epidermal fractions were immediately frozen in liquid nitrogen.

* One DNA sample was prepared from three pooled papillomas.

† Both DNA samples were prepared from 8–10 pooled papillomas.

‡ Samples positive in transfection assay.

with the exception of the RNA from the papilloma in lane e and from the TPA-treated epidermis, approximately equivalent amounts of undegraded RNA were present in each lane (data not shown). Similar relative transcript levels have been found using RNA dot hybridization with probes specific for *ras*^H, 7S RNA and ribosomal RNA sequences (data not shown). We conclude that *ras*^H transcripts are elevated to a varying degree in different papillomas. The elevation is not due simply to an increase in the proportion of proliferating cells within the tumours because the hyperplastic epidermis induced by TPA does not contain substantially higher *ras*^H transcript levels.

An increase in the concentration of *c-ras*^H transcripts with respect to NIH/3T3 controls was also observed in transformed foci induced by transfection of DNA from papillomas or carcinomas (Fig. 2B). No reproducible correlation was found between steady-state concentrations of *ras*^H-related transcripts and the source of DNA used to induce the transformants.

The elevation of *c-ras*^H transcripts which is observed in the chemically induced primary tumours contrasts with the observations reported on the transcription of the equivalent human gene in the EJ/T24 bladder carcinoma cell line. Tabin *et al.*¹⁶ compared *c-ras*^H transcript levels in EJ cells and in a normal human bladder cell line, and found no detectable differences. A possible explanation of this apparent contradiction is that the establishment of normal cells in culture leads to elevated *ras*^H gene expression, thus disguising any true increase during tumour development *in vivo*. In this context, it is interesting that elevated *ras*^H transcription has recently been found in several primary human tumours of the colorectum¹⁷. Alternatively, prolonged culture of tumour cells may result in lower levels of *ras*^H transcripts. It is also possible, if somewhat less likely, that differential extraction of RNA from basal or supra-basal cells could have occurred in our experiments, leading to artificially low *ras*^H transcript levels in control epidermis.

The number of independent genetic events involved in the transformation of a normal cell into a malignant tumour cell is unknown, but estimates range from two^{18,19} to about seven²⁰. It seems likely that the acquisition of dominant transforming properties by the DNA of malignant cells constitutes an important step in tumour progression. It has previously been speculated that this event occurs relatively late in tumour development²¹. This was based on the assumption that NIH/3T3 cells may already exist in an 'initiated' state, and require only one additional (late) event to acquire malignant properties. The data presented here, however, demonstrate that during chemical carcinogenesis *in vivo*, activation of the *ras*^H gene is a relatively

early event. The proportion of papilloma DNAs which exhibited transforming activity (>85%) is much higher than the percentage of benign tumours which progress to carcinomas in this system (5–7%)⁵. This indicates that the mutational event which induces the transforming capacity of the DNA does not take place at the transition to malignancy, but already exists in most or all of the pre-malignant lesions.

Our results demonstrate that no significant differences are found between papillomas and carcinomas with respect to transforming activity of high molecular weight DNA and the degree of transcription of the activated *c-ras*^H gene. The progression of some, but not all, papillomas to malignant carcinomas may therefore be determined by another genetic event which does not involve the *c-ras*^H gene.

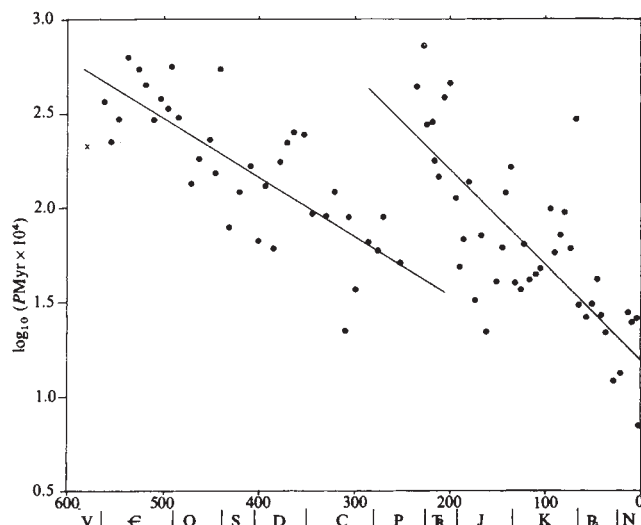
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- Pierce, G. B. & Fennell, R. H. in *Cancer Medicine* (eds Holland, J. F. & Frei, E.) 149–167 (Lea & Febiger, Philadelphia, 1982).
- Knudson, A. G. *Cancer Invest.* **1**, 187–193 (1983).
- Hecker, E., Fusenig, N. E., Kunz, W., Marks, F. & Thielmann, H. W. (eds) *Carcinogenesis* Vol. 7 (Raven, New York, 1982).
- Boutwell, R. K. *Crit. Rev. Tox.* **2**, 419–443 (1974).
- Burns, F. J., Vanderlaan, M., Snyder, E. & Albert, R. E. In *Carcinogenesis* Vol. 2 (eds Slaga, T. J., Sivak, A. & Boutwell, R. K.) 91–96 (Raven, New York, 1978).
- Cooper, G. M. *Science* **217**, 801–806 (1982).
- Balmain, A. & Pragnell, I. B. *Nature* **303**, 72–74 (1983).
- Digiovanni, J., Slaga, T. J. & Boutwell, R. K. *Carcinogenesis* **1**, 381–389 (1980).
- Hennings, H. *et al. Cancer Res.* **41**, 773–779 (1981).
- Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. *Nature* **298**, 343–347 (1982).
- Der, C. J., Kroniris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3637–3640 (1982).
- Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. *Nature* **297**, 474–478 (1982).
- Shimizu, K., Goldfarb, M., Perucho, M. & Wigler, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 383–387 (1983).
- Pulciani, S. *et al. Nature* **300**, 539–542 (1982).
- Balmain, A., Krumlauf, R., Vass, J. K. & Birnie, G. D. *Nucleic Acids Res.* **10**, 4259–4277 (1982).
- Tabin, C. J. *et al. Nature* **300**, 143–149 (1982).
- Spandidos, D. & Kerr, I. B. *Br. J. Cancer* (submitted).
- Armitage, P. & Doll, R. *Br. J. Cancer* **11**, 161–169 (1957).
- Moolgavkar, S. H. & Knudson, A. G. *J. natn. Cancer Inst.* **66**, 1037–1051 (1981).
- Cook, P. J., Doll, R. & Fellingham, S. A. *Int. J. Cancer* **4**, 93–112 (1969).
- Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. *Nature* **300**, 149–152 (1982).
- Ellis, R. W. *et al. J. Virol.* **36**, 408–420 (1980).

Errata

IN the letter 'A resetting of Phanerozoic community evolution' by L. M. Van Valen, *Nature* **307**, 50–52 (1984), the words 'It is therefore not feasible' in paragraph 5 should be replaced by 'This sort of analysis cannot be used'; and the reference to the 'Red Queen hypothesis' on page 51 should read 'Red Queen's hypothesis'. In addition, Fig. 1 was poorly reproduced: a correct version is shown below.



Lunar magnetism

In a recent review of lunar palaeomagnetic investigations, Runcorn¹ has emphasized the possibility that an ancient core dynamo was the dominant source of the observed crustal magnetization by generating surface fields of 0.01–1.0 G in the $3.2\text{--}4.0 \times 10^3$ Myr time period. Major implications for lunar internal and dynamical history were discussed in part on the basis of both palaeomagnetic data and electromagnetic sounding data. Here we emphasize aspects of the lunar palaeomagnetic and electromagnetic sounding results which appear to be inconsistent with the core dynamo hypothesis; a more complete discussion can be found elsewhere².

First, various geophysical measurements limit the radius of a present-day lunar metallic core to a maximum of 400–500 km and do not require a metallic core of any radius. Therefore, the statement “evidence for an iron core in the Moon today (has) been found” (ref. 1) is somewhat misleading. Low-frequency electromagnetic sounding measurements for times when the Moon was in the solar wind or terrestrial magnetosheath^{3,4} have been shown by Hobbs *et al.*⁵, using inverse theory, to imply only an upper bound of 435 km on the radius of a possible metallic core; the data are equally consistent with a core radius of 0 km. Although Russell *et al.*⁶, in an analysis of magnetometer records from periods when the Moon was in the lobes of the geomagnetic tail, obtained tentative evidence for a core with conductivity $\geq 10 \text{ S m}^{-1}$ and radius ≥ 400 km, a molten silicate core could equally well provide a conductivity of this magnitude so an iron core with conductivity $\geq 10^5 \text{ S m}^{-1}$ is not required. In addition, remaining questions about the possible effects of tail-lobe plasma on the induced moment measurements preclude acceptance of these results as definitive. Recent summaries of lunar seismic investigations conclude that seismic evidence for the existence of a metallic core is unresolved^{7,8} with earlier suggestions of a possible molten core of radius ≤ 350 km continuing to depend on the interpretation of a single data point⁹. Other independent constraints are model-dependent to equal or greater extents as noted by Runcorn.

Anderson¹⁰, using Busse's theory of planetary magnetism¹¹, has calculated that a lunar spin rate 20 times the present value combined with a fluid conducting core radius of 750 km would be necessary to produce surface palaeofields of 0.02 G, that is, approximately corresponding to minimum palaeointensities during the $3.2\text{--}4.0$ Myr time period. To produce surface fields of ~ 1 G, corresponding to the

high-field epoch discussed by Runcorn and Cisowski *et al.*¹², a core radius exceeding 1,000 km would be required. Therefore, assuming the first-order validity of Busse's model, the geophysical constraints on the radius of a metallic core summarized above are inconsistent with an iron core dynamo origin for lunar palaeomagnetism. In order to overcome these difficulties, Cisowski *et al.* have suggested a possible close approach of the Moon to the Earth at $\sim 3.8 \times 10^3$ Myr acting to amplify a marginal lunar dynamo according to a mechanism considered theoretically by Levy¹³. However, even accepting the latter hypothesis, it is doubtful that a core dynamo can explain all of the magnetization that is inferred from both orbital and sample studies to have occurred over a period exceeding 10^3 Myr. An additional mechanism is needed as has also been stressed by Anderson.

Circumstantial evidence for the identity of this mechanism comes from orbital magnetometer and electron-reflectance studies of the distribution of lunar magnetic anomalies and their correlation with surface geological features as well as from returned sample studies and surface magnetic field investigations. On the near side, most magnetic anomalies detected at low altitudes with the Apollo subsatellite magnetometers correlate with exposures of the Fra Mauro and Cayley Formations, primary and secondary basin ejecta materials¹⁴. Additional anomalies detected across part of the far side using the electron reflectance technique have been shown to occur peripheral to ringed impact basins where basin ejecta units are observed or are inferred to be present¹⁵. Two separate exposures of the Fra Mauro Formation peripheral to the Imbrium basin, separated by ~ 100 km, have been inferred to be magnetized in very different directions, indicating a local scale size for the magnetizing field¹⁶. High-amplitude orbital anomalies detected via both techniques generally correlate with surficial concentrations of apparently relatively young Reiner γ -type units which most probably have an impact-related origin^{14,17–19}. In combination with the relatively high intrinsic magnetization levels that are characteristic of some returned breccias and soil samples as compared with mare basalts^{20,21}, these correlations strongly suggest that surface ejecta materials produced in large-scale meteoroid impacts are primary sources of the observed orbital magnetic anomalies. The lack of large-scale anomalies associated with major structural features such as the mascons argues against thermal remanence or chemical remanence acquisition by thick crustal layers cooling over long time periods in the presence of a

large-scale steady magnetic field. The lack of clear evidence for thermal remanence acquisition for at least some fine-grained mare basalts²² and the 0.025 G Thellier palaeointensity obtained for a young (< 200 Myr) Apollo 17 impact-produced glass sample²³ are also problematical or contradictory observations for the core dynamo hypothesis.

Because many orbital anomalies are closely associated with relatively surficial impact-produced materials and since impacts are the dominant sources of localized, near-surface energy during lunar history, an impact-related origin for lunar magnetizing fields has been repeatedly proposed. The possibility that transient magnetic fields may be generated as a result of the interaction of highly electrically conducting ionized ejecta with a weak ambient interplanetary magnetic field was first suggested by Hide²⁴ with specific reference to lunar palaeomagnetism. Spontaneous generation of transient magnetic fields in energetic plasma clouds produced in hypervelocity meteoroid impacts was later suggested by Srnka²⁵ based on theoretical models for magnetic field observed in laser-heated plasmas. More recently, Cerroni and Martelli²⁶ have theoretically investigated field generation by thermally driven currents and compression of a pre-existing magnetic field at the boundary of an expanding ionized ejecta cloud with application to lunar palaeomagnetism. Field compression mechanisms involving cometary impacts have also been proposed on several occasions^{17,27}. Although the kinetic energies of major impacts on planetary surfaces far exceed those achievable in laboratory experiments, some progress has been made towards experimental detection of plasma, magnetic field and rock magnetization effects associated with hypervelocity impacts onto basalt targets^{28–30}. In this context, it is of interest that lunar palaeointensity data indicate that the more intense palaeofields (~ 1 G) were present near the termination of the basin-forming epoch¹². Since impact-generated fields are expected to scale as a positive power of the scale of the impact and since the basin-forming impacts were the most energetic in lunar history, this result may support the impact model rather than refute it as was argued by Runcorn.

Thus, while it is not yet possible clearly to identify the mechanism(s) responsible for generation of strong magnetic fields at the lunar surface implied by the Apollo palaeomagnetic investigations, the possible importance of local mechanisms involving meteoroid impact processes is suggested by a large fraction of the available evidence. In such conditions, it is

advisable to consider cautiously the proposed implications of a former lunar dynamo.

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Contribution No. 523.

1. Runcorn, S. K. *Nature* **304**, 589–596 (1963).
2. Hood, L. & Cisowski, S. *Rev. Geophys. Space Phys.* **21**, 676–684 (1983).
3. Hood, L. L., Herbert, F. & Sonett, C. P. *J. geophys. Res.* **87**, 5311–5326 (1982).
4. Sonett, C. P. *Rev. Geophys. Space Phys.* **20**, 411–455 (1982).
5. Hobbs, B., Hood, L., Herbert, F. & Sonett, C., *J. geophys. Res.* (in the press).
6. Russell, C. T., Coleman, P. J. & Goldstein, B. E., *Geochim. cosmochim. Acta Suppl.* **12**, 831–836 (1981).
7. Goins, N. R., Toksoz, M. N. & Dainty, A. M. *Geochim. cosmochim. Acta Suppl.* **9**, 2421–2439 (1979).
8. Nakamura, Y., Latham, G. V. & Dorman, H. J. *J. geophys. Res.* **87**, A117–A123 (1982).
9. Nakamura, Y. *et al. Geophys. Res. Lett.* **1**, 137–141 (1974).
10. Anderson, K. A. *J. geophys. Res.* **88**, A588–A590 (1983).
11. Busse, F. *Ann. Rev. Fluid Mech.* **10**, 435–462 (1978).
12. Cisowski, S. M., Collinson, D. W., Runcorn, S. K., Stephenson, A. & Fuller, M. *J. geophys. Res.* **88**, A691–A704 (1983).
13. Levy, E. H. *Geochim. cosmochim. Acta Suppl.* **10**, 2335–2342 (1979).
14. Hood, L. L., Coleman, P. J. & Wilhelm, D. E. *Science* **204**, 53–57 (1979); *Geochim. cosmochim. Acta Suppl.* **10**, 2235–2257 (1979).
15. Anderson, K. A. & Wilhelm, D. E. *Earth planet. Sci. Lett.* **46**, 107–112 (1979).
16. Hood, L. L. *Geochim. cosmochim. Acta Suppl.* **11**, 1879–1896 (1980).
17. Schultz, P. H. & Srnka, L. J. *Nature* **248**, 22–26 (1980).
18. Hood, L. L. *Nature* **287**, 86 (1980); *Geochim. cosmochim. Acta Suppl.* **12**, 817–839 (1981).
19. Hood, L. L., Lin, R. P. & Metzger, A. E. *Lunar planet. Sci.* **14**, 323–324 (1983).
20. Strangway, D. W., Gose, W., Pearce, G. & McConnell, R. K. *Nature* **246**, 112–113 (1973).
21. Fuller, M. *Rev. Geophys. Space Phys.* **12**, 23–70 (1974).
22. Fuller, M., Cisowski, S. M. & Hale, C. J. *Geochim. cosmochim. Acta Suppl.* **10**, 2211–2233 (1979).
23. Sugiura, N. *et al. Geochim. cosmochim. Acta Suppl.* **10**, 2189–2198 (1979).
24. Hide, R. *The Moon* **4**, 39 (1972).
25. Srnka, L. J. *Geochim. cosmochim. Acta Suppl.* **8**, 785–792 (1977).
26. Cerroni, P. & Martelli, G. *Planet. Space Sci.* **30**, 395–398 (1982).
27. Gold, T. & Soter, S. *Planet. Space Sci.* **24**, 45–54 (1976).
28. Martelli, G. & Newton, G. *Nature* **269**, 478–480 (1977).
29. Srnka, L. J. *et al. Earth planet. Sci. Lett.* **42**, 127–137 (1979).
30. Bianchi, R. *et al. Nature* (submitted).

RUNCORN REPLIES—Hood, Sonett and Srnka's conclusion that "the possible importance of local mechanisms involving meteoroid impact processes is suggested" largely rests (in their two penultimate paragraphs) on the observed correlation between the magnetic anomalies, mapped by the subsatellite magnetometers and by the reflected electron technique, and the ejecta blankets from impact events. This argument is scarcely logical: one might just as well argue that as lava flows are the most strongly magnetized rocks on the Earth and that as volcanoes are "the dominant sources of localized near surface

energy during (Earth) history" the remanent magnetization of lavas must be produced by electric phenomena in the eruptions and has nothing to do with the geomagnetic field.

Hood *et al.* refer to two sources 100 km apart with "very different directions of magnetizations"; my Fig. 3b (ref. 1) shows that these (C1, C2) are roughly opposite. I explained that this is entirely consistent with the ejecta having a uniform direction of magnetization on an even larger scale. The ejecta, like the lavas, can only become magnetized with uniform direction after final emplacement in the presence of a uniform ambient field. No experimental demonstration has yet been made of magnetization produced during hypervelocity impacts on basalts that is attributable to transient plasma fields rather than the Earth's field, but such processes may well account for some magnetized material on the Moon, and elsewhere in the Solar System. My review was concerned with the test of the rival hypotheses. The bipolar clustering of north magnetic poles along three different axes, the proximity of the multi-ring basins to, and the parallelism of the velocities of the bodies that created them with the palaeoequators of corresponding age, strongly support magnetization by an ancient core dynamo field and cannot be understood on the magnetization by impact hypothesis. In Table 1 (ref. 1), the axis of rotation for the mean poles of Lower Nectarian age should be 50.4° S 162° E and for the Pre-Nectarian poles 7.8° N and 92.3° E.

That the lavas in the maria basins, especially the thick ones in the circular maria (which account for the mascons), do not show up by the magnetic mapping is not a mystery as we have Apollo lava samples and their intensity of magnetization is 10^{-5} EMU cm⁻³—an order of magnitude less than that of breccia. Such uniformly magnetized layers of lava produce less than 0.1 nT near their surfaces, except at their edges where the fields are about 10 nT but fall off so rapidly with height as to be undetectable by the subsatellite. By contrast, the magnetic effect of the Rima Sirsalis graben feature 10 km across and 300 km long has been detected^{2,3}. That the intensity of magnetization of ejecta and breccia is an order of magnitude greater than that of lavas is explained by difference in the amount of free iron: it seems likely⁴ that in the impacts reduction of silicates occurs due to rise in temperature with the release of Fe metal grains.

Since I wrote my review, two further lines of evidence for a lunar iron core have been found. First, lunar laser ranging has determined that the axis of rotation makes an angle of 0.23 arcs with the Cassini plane, the mean dissipation within the Moon corresponds to $Q=28$. Seismic studies show that the Q of the outer parts of the Moon is 1,000, so Yoder⁵ interprets the result in terms of an iron core. Second, Newsom and Drake⁶ find that tungsten is

depleted more in the lunar rocks than in the Earth's mantle, where it is 10^{-4} of Solar System abundances. As the latter depletion is ascribed to the Earth's core and as general abundance patterns between the Earth and Moon are similar, indicating a common origin, a further extraction of siderophile elements is required for the Moon: thus Newsom and Drake argue for an iron core. I wrote about the (now six) independent lines of evidence: "None of these is conclusive evidence for an iron core, in each case other models can be generated to fit the observations"; thus the free iron Drake and Newsom require might be distributed throughout the Moon like currants in a cake. That Hood *et al.* consider my account as "somewhat misleading" can only mean that they do not appreciate that even though each line of evidence is not conclusive the fact that all point to the existence of a lunar core lends much greater weight to the hypothesis than any one line could: a venerable principle of reasoning called Occam's razor. In fact other models would be mutually inconsistent: the 'currants in the cake' model would not explain the low moment of inertia or the delayed seismic P wave.

Anderson's argument is based on Busse's theory in which the strength of a dynamo field is independent of the energy source driving the convection: experiments on dynamos and all other theories gave a contrary result⁷. Busse's result was obtained by equating the Coriolis term and the Lorentz force in the dynamo equation. I conclude that the dynamo theory, which in spite of its attractiveness did not in fact predict any of the many phenomena discovered in Earth and planetary magnetic fields, is not understood sufficiently well to be a secure basis for Anderson's argument.

S. K. RUNCORN

1. Runcorn, S. K. *Nature* **304**, 589–596 (1983).
2. Anderson, K. A., Lin, R. P., McGuire, R. E. & McCoy, J. E. *Earth planet. Sci. Lett.* **34**, 141–151 (1977).
3. Srnka, L. J., Hoyt, J. L., Harvey, J. V. S. & McCoy, J. E. *Phys. Earth planet. Inter.* **20**, 281–290 (1979).
4. King, E. A. *J. geophys. Res.* **87**, Suppl., A429–A434 (1982).
5. Yoder, C. F. *Phil. Trans. R. Soc. A* **303**, 327–338 (1981).
6. Newsom, H. E. & Drake, M. J. *Nature* **297**, 210–212 (1982).
7. Soward, A. M. & Roberts, P. H. *Rotating Fluids in Geophysics* (Academic, New York, 1974).

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Turing and the computer

I.J. Good

Alan Turing: The Enigma. By Andrew Hodges.

Burnett Books (distributed by Hutchinson)/Simon & Schuster: 1983.
Pp.587. £18, \$21.75.

A SHORT biography of Alan Turing was written by his mother but this new one, by Andrew Hodges, is much more complete, objective and illuminating. Turing was one of the pioneers of computer science. His most notable work in this area was related to the foundations of mathematics, appreciation of which requires some relevant background.

A set whose elements can be put into one-to-one correspondence with the positive integers 1, 2, 3, . . . is said to be *countable*. For example, the even numbers 2, 4, 6, . . . form a countable set. The mathematician David Hilbert invented a hotel in which the rooms were numbered 1, 2, 3, . . . to infinity. One day all the rooms were occupied, but a countable number of additional people wanted rooms. So, for each n , the resident in room n moved to room $2n$, thus vacating the odd-numbered rooms for accommodating the newcomers.

A number of the form m/n , where m and n are integers, is said to be *rational*. At first sight, the set of rational numbers seems to be "larger" than the set of integers, but it is still countable. For we can imagine the positive rational numbers "counted" in the order $1/1$; $2/1$; $1/2$; $3/1$; $2/2$; $1/3$; $4/1$; $3/2$; $2/3$; $1/4$; $5/1$, This order fills successively larger triangles if m/n is written at a point with coordinates (m, n) . Every positive rational number will ultimately get counted, in fact repeatedly. We might now wonder whether *every* set is countable, but Georg Cantor showed, in about 1880, that the set of "real" numbers is not countable. A real number can be written as an infinite decimal; for example, $\pi = 3.1415926 \dots$ is a real number. We now show that the real numbers between 0 and 1 are uncountable by assuming that they *are* countable and then deducing a contradiction.

Imagine a sheet of squared paper having a top left-hand corner and extending infinitely to the right and downwards. Suppose the real decimals between 0 and 1 can be listed in the order $x_1, x_2, x_3, \dots$. (We are not assuming, for example, that $x_1 < x_2 < x_3 < \dots$ or that $x_1 > x_2 > \dots$.) Let the digits of x_1 be written on the first row of the squared paper, the digits of x_2 on the second row, and so on. Now define a new decimal y whose n th digit (for each n) is not the same as the n th digit of x_n . Clearly y is not equal to x_n for any n . So there is a real number y not on the list and this contradicts our assumption that all the real numbers were on the list. Thus the real numbers are uncountable. (I have ignored

the unimportant ambiguous notation for terminating decimals such as .9999) This argument is called Cantor's *diagonal argument* and it shows that infinite sets are not all of the same "size". Some great mathematicians at the time called Cantor's work theology because they were unwilling to admit he was a genius.

The diagonal argument has since been used, in a very much more sophisticated

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Alan Turing, at the time of his election to the Fellowship of the Royal Society in 1951.

manner, to prove remarkable results in the logic of mathematics. The first example was published by Kurt Gödel in 1931. He showed that no finite set of axioms about the integers could capture all their properties. In other words, given a finite set of axioms, and a precisely specified definition of what is meant by a proof, there are statements in the theory of numbers that are true but cannot be proved.

One might naively assume that the *meaning* of the truth of a mathematical statement is that it follows from the axioms. But this assumption can be seen to be dubious by means of the following simple example. Consider the conjecture that π contains arbitrarily long runs of 7s in its decimal expansion. For the sake of argument, suppose that this conjecture is true. Then, for each positive integer n , one could in principle prove that the decimal expansion contains runs of n consecutive 7s by straightforward computation. (When a mathematician says that something can be done "in principle" he usually knows that it is impracticable.) But it might not be possible to prove the conjecture itself because a proof must, by definition, be of finite length.

We are now ready to understand broadly

what Turing discovered in 1935 when he was only 22. He first defined rigorously a "paper machine" capable of carrying out any conceivable calculation when suitably programmed, a concept now called a "Turing machine" by computer scientists. General-purpose electronic computers are approximately equivalent to Turing machines. He then defined a computable (real) number x as one for which a program (of finite length) could be written that would compute more and more digits of x . Since the number of possible programs is only countably infinite, and only some of them give computable numbers as output, it follows that the number of computable numbers is also only countably infinite. But Turing showed that there is no *explicit* way to do the counting. For suppose it is possible for a master program to have as its output a list of all the computable numbers, on squared paper, x_1 in row 1, x_2 in row 2, etc. Of course the computer would not list the whole of x_1 before starting on x_2 , etc. It might, for example, print in order $x_{11}, x_{21}, x_{12}, x_{31}, x_{22}, x_{13}$, etc., where x_{ij} is the j th digit of the computable number x_i . (Compare the triangular process for "counting" the rational numbers.) But then one could define a *new* computable number by means of the diagonal process. So the alleged master program is not really a master program at all — and this completes the proof of Turing's result by *reductio ad absurdum*. From this result he was able to deduce that there is no automatic procedure, using only the rules of a given formal system, that can decide, for every mathematical statement, whether it is provable, unprovable or neither. In the book, Andrew Hodges provides a rigorous account of this work.

During the Second World War Turing worked on the cryptanalysis of the Enigma, a German cryptographic machine. He had an important idea for improving the "Bombe", which was the main cryptanalytic machine used for attacking the Enigma. The idea was based on the logical fact that from a contradiction anything can be proved, a fact that Wittgenstein thought was unimportant. It saved a vital factor of about 26 in the running time, and if it had not been for that the Allies might have lost the war. Turing also made important statistical contributions, but had little to do with the Colossus, a cryptanalytic machine which, unlike the Bombe, had much in common with later electronic computers.

Immediately after the War Turing worked on the design of the ACE (Automatic Computing Enigma) at the National Physical Laboratory (NPL), a post he took partly because he did not enjoy lecturing. The storage of the ACE was based on mercury delay lines; and its programming, as M.H.A. Newman said, was like catching mice just as they were entering a hole in the wall. Some machines with magnetic drums had the same disadvantage. Turing was not happy at NPL,

because he was not allowed to be both a mathematician and an engineer, and in 1947 he took a year's leave during which he wrote an influential philosophical paper on artificial intelligence. In this article he disposed of most of the arguments that are made against the possibility of the machine simulation of thought processes. In 1948 he moved to Manchester and produced the programming system for the Manchester University Computer, Mark II.

Turing was logical but emotional, excitable but kind, eccentric but natural, and intellectually and physically strong. He was a very good long-distance runner and



Turing, the runner, in the mid-1940s.

might have run in the Olympic games, but developed a neurological complaint in a leg. After the war he admitted privately that he ran partly to work off libido and that he was a homosexual; fortunately M15 did not know of his homosexuality during the war. In 1952 he published a seminal contribution to the mathematical theory of growth, but in the same year he was convicted for having a homosexual relationship with a 19-year-old man and was put on probation and on chemical therapy. In June 1954 Turing committed suicide.

In this biography Hodges treats most of these aspects of Turing's life, and many others, and attempts to trace the origins of his ideas in his early environment. The book is researched and written extraordinarily well; I found only a few minor inaccuracies in spite of having known over a hundred of the people mentioned by Hodges. It is a first-class contribution to history and an exemplary work of biography. □

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Formats for language development

John McShane

Child's Talk: Learning to Use Language.

By Jerome Bruner.

W.W. Norton/Oxford University Press: 1983. Pp.144. Hbk \$13.95, £11; pbk £5.95.

A CHILD must master a complex grammatical system in acquiring language. How he does so has been a central issue of debate and research for the past twenty-five years. Much of the discussion has concerned what sort of unique language-learning capacities human beings might or might not have. However that question is answered eventually, a child would not learn a language without being part of a community of other speakers.

Thus, any reasonably complete answer to the question of language acquisition must give an account of both the child's language-learning capacities and the environmental features that stimulate those capacities. Bruner's book is a summary of a decade of his work on the role of the environment in language acquisition. It is not a general review of research in this area; indeed, there is little mention of some standard issues such as the grammatical characteristics of parental talk to children. Instead, problems that have concerned Bruner during the past decade are examined, considered and summarized. The book provides a clear and illuminating introduction to these concerns but, inevitably perhaps, fails to satisfy one's desire for a thorough-going consideration of the positions of other theorists.

Chapter 2, which deals with the relations between language and non-language, is the clearest example of where more was needed. Bruner makes two points concerning the non-linguistic foundations of language. The first is that language structures have analogues in other areas of cognition. Thus, at least some of the structure of language might be derived non-uniquely. The trouble with this view is that it is notoriously difficult to move from pointing at plausible candidates from which to derive grammatical structures to demonstrating a causal link, as Bruner recognizes. Because he has not set out to write a detailed critical review, these complex issues are finessed. Bruner's other point is that a child is not simply learning grammatical structures in learning a language; he is also learning the conventions for the use of linguistic structures. He argues forcefully that many of these conventions are set-up by the child's social environment, independently of the child's grammatical abilities and usually well in advance of them. The

mechanism by which this occurs is the heart of the book.

Bruner argues that adults create structured interactions with children and maintain the basic structure over many months while gradually handing over more and more control of the structure of the interaction to the child. Bruner calls these structures *formats*. Within a format a child gradually achieves an increasing degree of competence with the adult's aid and cajoling. As competence increases so the adult demands for competence increase. Formats for peekaboo games, reference and requesting are considered in some detail.

Not many researchers have followed Bruner's path of exploring the *interaction* of adult and child in detail and implicating the structure of the interaction in a causal model of development. To my mind this is a great pity. While this particular tack does not answer all questions about language development, it seems to be a more fruitful approach to the role of the environment than the more common technique of correlating features of parental speech with later features of the child's speech, in complete oblivion of the interactional context in which the speech occurred. The more dynamic approach of Bruner is both time-consuming and labour-intensive on the part of the researcher. This book makes a sound case for the investment required. □

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Control by calcium

P.F. Baker

Intracellular Calcium: Its Universal Role as Regulator.

By Anthony K. Campbell.

Wiley: 1983. Pp.556. £36.75, \$79.95.

THE importance of intracellular calcium as a widespread, if not universal, regulator of cell function is well recognized. "Control by calcium" exhibits three key features: maintenance of a very low cytosolic free Ca in the face of intracellular and extracellular pools of Ca at much higher concentrations; the presence of special voltage or receptor-controlled mechanisms for permitting Ca to enter the cytosol from one or more of these high Ca pools; and the existence within the cytosol of a special class of Ca-receptor proteins that serve to transduce alterations in free Ca to changes in enzyme activity and cell function. Despite this apparent simplicity, it is often difficult in practice to unravel the part played by calcium in a particular physiological process and much effort has been expended in trying to develop suitable experimental techniques. The success of this effort is

now reflected in an avalanche of publications in the primary literature.

When new material is appearing at such an alarming rate, to embark on a book can be a dangerous decision. Exceptions are books that aim to impose some sort of order on the apparent chaos of the primary literature and those that advance a novel framework within which the new discoveries can be set. Tony Campbell's *Intracellular Calcium* falls squarely into the first category.

After discussing the "Natural History of Calcium" and "Calcium Methodology", Campbell sets out to draw together the arguments for and against a role for Ca in a host of cellular processes with chapters on "Intracellular Ca and the Electrical Activity of Cells", "Calcium and Cell Movement", "Intracellular Ca and Intermediary Metabolism", "Endocytosis and Exocytosis", "The Reproduction and Development of Cells" and "The Pathology and Pharmacology of Intracellular Ca". Each of these often contains long accounts of basic cell biology as an essential introduction to discussion of the possible roles of calcium. Unfortunately, except where that role is already well known, these chapters often end rather inconclusively. They nevertheless certainly highlight many unsolved problems of cell biology in which calcium may well be involved.

Such a wide sweep has, however, posed obvious difficulties for the author and despite 556 pages the book falls somewhere between being a natural history of intracellular calcium and a critical discussion of those processes where involvement of calcium is still controversial. In consequence some important growth areas of real significance to "control by calcium" are under-represented and presented in a relatively uncritical fashion. Two obvious examples are phosphoinositol metabolism and the transport of calcium across membranes; but I suspect that most specialists will find fault with the presentation of their own particular neck of the calcium wood. It is a pity that the author did not seek more feedback before publication. In this way the most glaring errors might have been avoided and the author might also have been encouraged to reference all the most important observations with original papers. As it is, the policy is mixed: sometimes original papers are quoted, in other places reviews, but all too often interesting observations go incognito.

It is all too easy to find fault. This is a very personal account of the many roles of intracellular calcium that will be an obvious starting point for those entering the field. It will also, I feel sure, infect both the old hand and the new with some of Tony Campbell's great enthusiasm for his subject. □

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Look back to viral oncology

Robin Weiss

Oncogenic Viruses, 3rd Edn.

By Ludwik Gross.

Pergamon: 1983. Two volumes, pp. 1,203. £100, \$200.

LUDWIK Gross has influenced the development of experimental oncology in the post-War period through three distinct contributions. First, after many years of frustrating experimental work, in 1951 he showed that the thymic lymphoma of AKR mice could be transmitted to new-born C3H mice by cell-free filtrates; this discovery of murine leukaemia virus, following Bittner's demonstration of the milk factor causing mammary carcinoma in mice 15 years earlier, firmly established that viruses cause cancer in mice, and led to a resurgence of tumour virus research. Second, in 1953, Gross reported that salivary gland tumours (also induced by the thymoma extracts) were caused by a distinct agent, later named polyoma virus by Sarah Stewart, who independently discovered the virus in the same year. Finally, in 1961, Gross published the first book wholly devoted to tumour viruses.

Twenty years ago Gross's *Oncogenic Viruses* was the only comprehensive general text available. As such, the book was invaluable. A second enlarged edition was published in 1970 which included much new material, though it omitted discussion of some of the most interesting *in vitro* experimentation on oncogenic viruses and cell transformation established during the 1960s. Now Gross has produced a third edition, expanded to two volumes, and including some new chapters on oncogenic viruses of fish, "slow virus diseases" and iatrogenic transmission of human cancer.

I looked forward to reading this new edition in the hope of seeing the recent strides made in experimental oncology placed in perspective by the man who discovered two of the most intensively studied tumour viruses. I was deeply disappointed. Whilst Gross writes in his introduction that "all chapters . . . have been brought up-to-date in order to include the most important observations which have become available since the previous edition was prepared", this is a travesty of what follows. For instance, there is not a single citation of David Baltimore's work, either on reverse transcriptase (which is never mentioned), or on Abelson leukaemia virus (this important virus and its associated neoplasms are dismissed in a single paragraph).

Gross excludes any discussion of viral replication and genetics. This could be explained by his remark that "molecular biology is not discussed in this book". But while the author wisely did not aim to write

a detailed molecular treatise, no modern text can afford to ignore the results of molecular biology in changing our conception of tumour virus biology, and indeed of cancer as a whole. It is difficult to see how any author can claim to present insights into viral oncogenesis without at least a nodding reference to "early" functions of papovaviruses or to retrovirus oncogenes.

By eschewing all data on viral and host genetics, whether obtained by classical or molecular methods, much of value in the detailed descriptions of early virus isolates is diminished. Such important topics as viral transmission, defectiveness, and host permissiveness and resistance are discussed in terms of concepts that were already outdated at the time of the second edition 13 years ago. For example Gross himself coined the terms "vertical" and "horizontal" transmission for tumour viruses in 1944, and the existence of hereditary infections is described at length in many chapters. Yet the crucial distinction between congenital infection and the Mendelian inheritance of integrated proviruses is not made until the final chapter, almost as a footnote. Classical papers, such as those by Payne and Chubb (*J. Gen. Virol.* 3, 379; 1968) on the Mendelian inheritance of viral antigens in chickens and Bentvelzen and Daams (*J. Natl Cancer Inst.* 43, 1025; 1969) on the chromosomal transmission of mammary tumour virus genomes in GR mice are not cited, let alone the wide body of knowledge on endogenous viral elements accrued in the 1970s which clarified the whole subject of retrovirus latency and activation. Gross devotes five chapters to murine leukaemogenesis without once mentioning xenotropic or recombinant viruses.

The considerable progress in the study of human oncogenic viruses since the last edition receives erratic treatment. With the burgeoning interest in human papilloma viruses and the isolation of new strains associated with particular neoplasms, it is astonishing to find the most recent reference dates from 1965. Hepatitis B viruses of humans and animals are summarized in one-and-a-half pages, and human T-cell leukaemia virus in one page plus an electron micrograph. There is, however, a whole chapter on Epstein-Barr virus, and a useful new chapter on "Inadvertent and Experimental Transmission of Human Cancer".

As an historical survey of viral oncology up to 1965, *Oncogenic Viruses* is unparalleled. But no reader or librarian should suppose that this new edition gives a contemporary account of viral oncology. As with Darwin's *Origin of Species*, I shall treasure the earlier editions, and regret that Gross has devoted much effort on a third edition which should not have been published. □

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The foraminifera in time

Gerta Keller

Neogene Planktonic Foraminifera: A Phylogenetic Atlas.

By James P. Kennett and M.S. Srinivasan.

Hutchinson Ross; 1983. Pp.265. \$36.50, £35.40.

A COMPREHENSIVE manual for the identification of Neogene planktonic foraminifera has been needed for some time. With this publication, James P. Kennett and M.S. Srinivasan have gone a long way to satisfying the need. The book contains high-quality scanning electron micrographs of 150 species which show the characteristic morphologies of type species as well as differences in surface ultrastructure. Each illustration is accompanied by a concise description of the species, and a note of its stratigraphic range and locality; a phylogeny based on morphological features is proposed for each lineage.

The book is especially noteworthy for the authors' representation of planktonic foraminifera by phylogenetic groups and for their erection of several new subgenera to distinguish evolutionary taxonomic lineages. This approach is both novel and useful for students, but unfortunately the subject is treated in a rather cursory manner. Apart from general comments in the introductory chapter there is little discussion of the proposed lineages and ancestor-descendant relationships, or of their controversial nature: ancestor-descendant relationships for many species are *not* well understood and, as noted by the authors, at present the use of phylogenetically defined subgenera is limited to themselves and one other worker.

There are a few minor, but important omissions of data on diachroneity of species ranges in the taxonomic descriptions. Species ranges (first and last appearances) are critical for the chronostratigraphic interpretation of sedimentary sequences. Therefore, care should be taken to note maximum species ranges (usually in the tropics) as well as known diachronous ranges of many species between tropical and temperate regions. The authors sometimes fail in this respect. For instance, *Globorotalia plesiotumida* (which defines the base of Zone N17) is diachronous even between the east and west equatorial Pacific, appearing at 9 Myr in the west and at 8 Myr in the east. For this reason, the first appearances of *Globigerinoides conglobatus* and *G. obliquus extremus* are more reliable in defining the base of Zone N17. Similarly, *Globoquadrina dehiscens* disappears in the tropics at 5 Myr, but at 10 Myr in temperate regions.

Some other species' ranges listed are also

anomalous. For example, *Globorotalia birnagae* and *Globigerinoides bolli* are listed as first appearing in Zones N7 and N12 respectively. However, both species have been reported from the early Miocene Zone N4. *Globigerinoides bulloideus* which is reported as first appearing in the late Miocene Zone N17 actually evolves in the middle Miocene. Most of these differences probably stem from the fact that the authors' synthesis is based primarily on investigations of deep-sea sequences from the south-west Pacific which, contrary to their claim, are not representative of the world oceans. Students should be aware of such problems and consult recent reports of the Deep Sea Drilling Project and micropalaeontological journals as appropriate.

While I have dwelt on the shortcomings, *Neogene Planktonic Foraminifera* is a well-conceived book for which the authors are to be thanked. It is an extremely handy manual for species identification and biostratigraphic interpretation which will prove invaluable to research workers, and to teachers and their students. □

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In the beginning

A.G. Cairns-Smith

Earth's Earliest Biosphere: Its Origin and Evolution.

Edited by J. William Schopf.

Princeton University Press; 1983. Pp.543. Hbk \$95, £88; pbk \$42.50, £40.

THE Precambrian Paleobiology Research Group was a realized pipe dream of J.W. Schopf's. It consisted of 24 scientists of appropriately diverse callings brought together to work, at UCLA and elsewhere, between May 1979 and August 1980. They engaged in research, in the field and in the laboratory; they met frequently, they talked and they published more than a dozen papers. And now, as the main product of their efforts, there is this splendid book.

The book includes the practical results of the group's activities, but it is not merely a series of research papers — rather, it is more an advanced textbook. The chapters are mainly reviews, but in spite of there being 21 contributors are reasonably well integrated. This was all helped no doubt by the editor's active authorship in almost half of them.

Preston Cloud was invited to introduce the book with a perspective on what he calls the new paradigm of biogeologic history — a mid-twentieth-century move from a too devout Uniformitarianism. The Earth is seen as having been substantially different in its early days, with a change through its history from reducing to oxidizing con-

ditions — a somewhat spasmodic change, though, with atmosphere, hydrosphere, biosphere and lithosphere evolving in concert.

A radical version of this new paradigm starts with the Oparin-Urey highly reducing atmosphere for the early Earth, a $\text{CH}_4\text{-NH}_3\text{-H}_2\text{O}$ type. Under such an atmosphere the probiotic synthesis of organic molecules should have taken place easily. Sherwood Chang *et al.* discuss in detail the question of the character of the probiotic atmosphere, believing that a build-up of organic molecules on the primitive Earth was a necessary preliminary to the start of a biological evolution. The strongly reducing first atmosphere is now seen as only one possibility — and perhaps not the most likely. The difficulty in deciding this question comes from trying to determine sequences of events during the formation of the Earth itself, some 4.5 Gyr ago. It is now generally thought that the early atmosphere was derived mainly from outgassing of the Earth. The nature of this atmosphere would then have depended on how much metallic iron there had been near the surface of the Earth, and that would have depended on how and when the core of the Earth was formed. These matters are also discussed in a chapter by David J. Stevenson, and they form the starting point for a discussion of the evolution of the lithosphere by W. Gary Ernst. The core formed early on, it seems, and most of the outgassing was early as well.

So after a somewhat catastrophic start there is, after all, a more Uniformitarian picture of the early Earth, according to several of the authors here — but with some gradual changes superimposed, needless to say. James C.G. Walker *et al.* take such a view. By the time there was a stable surface — and certainly by 3.8 Gyr ago when the most ancient known sediments were formed — the Earth was not so vastly different a place from now. By that time, it seems, the atmosphere was basically $\text{CO}_2\text{-N}_2\text{-H}_2\text{O}$ (whether or not it had ever been anything else) and evidently weathering and sedimentation processes were already in action. Walker and his colleagues make a plausible case for there having been much more carbon dioxide though — perhaps 100 times as much as now — and there is ample evidence for low or negligible oxygen levels.

Most people think that the appearance of significant amounts of oxygen in the atmosphere (very roughly 2 Gyr ago) was due to increasing photosynthetic activity by organisms. Jan Veizer suggests that organisms had perhaps been producing oxygen in quantity long beforehand and it was the gradual evolution of the lithosphere that brought about this atmospheric change: the Earth was cooling; reducing power derived from the mantle (e.g. Fe^{2+}) was being less vigorously injected into ocean waters and could no longer keep pace with the photosynthetic oxygen pro-

duction. . . . A neat inversion, this, of the more usual idea that evolutionary biology is the gradualist science and geological history the Uniformitarian one. Indeed ^{13}C studies on ancient sediments, reviewed by Shidlowksi *et al.*, fit perfectly well with the notion that a biosphere of similar scale to today was already present 3.5, possibly 3.8 Gyr ago: there is a typical biological (autotrophic) signature in the isotope ratios of reduced and oxidized materials, although it must be said that such signatures can be forged by non-biological processes.

Chapters by Schopf, Malcolm R. Walter and Hans J. Hoffmann on Precambrian fossils serve to underline the stability of many biological forms. Not all that much seems to have been happening between 3.5 Gyr ago and towards the end of the Precambrian (0.57 Gyr ago) when truly multicellular forms of life came on the scene. The 3.5 Gyr date is of rocks from Western Australia that contain fossil stromatolites (microbial colonies) as well as fossils of filamentous bacteria similar to modern members of stromatolite communities. To be told that of 43 cases of pre-2.5-Gyr-old sets of "microfossil-like objects" there are only two that are well proven, is a little worrying — one wonders whether more work might cast doubt here too. Well, there is no absolute security in this field and the evidence seems pretty good to me. If it holds it is the nearest we have yet got to the origin of life (although still not very near if these are fossils of anything at all like modern prokaryotes).

Past sequences of events within the biosphere can perhaps be inferred from the ways in which present biochemical subsystems depend on each other. Howard Gest and Schopf see sugars as primary materials, with fermentation and then (non-oxygenic) photosynthesis having been achieved early on (by 3.5 Gyr ago on the basis of isotope and fossil evidence). J.M. Hayes makes a case for oxygen-producing photosynthesis having appeared by about 2.8 Gyr ago: a wide spread of carbon isotope ratios in reduced materials of that sort of age fits with the idea that around then both oxygen-rich and oxygen-poor sedimentary basins were common. Whatever the exact timing, the more general oxygenation of the environment is seen by David J. Chapman and Schopf to have left its mark in the structures of modern biochemical pathways which indicate a progression from our remoter ancestors, which could not put up with oxygen, through forms that could tolerate it, to those that needed oxygen to survive.

This is a good-looking book with plenty of data and models attractively presented in pictures, diagrams and tables. I can see it in studies, in laboratories and even on coffee tables. It should help to generate some new addicts for an untidy, unsafe but exciting field. □

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Astronomical city

David W. Hughes

The Story of Astronomy in Edinburgh.

By Hermann A. Brück.

Edinburgh University Press: 1983.

Pp. 151. £8.50.

EDINBURGH is the home of a famous university and a Royal Observatory, and the history of astronomy in that city is a tale of the intricate symbiotic relationship between the two establishments. Both are old. Nineteen-eighty-three saw the 400th anniversary of the University and 1972 the 150th anniversary of the Royal Observatory, so it is a fitting time to delve into the development of astronomy in the city. Between the years of 1957 and 1975 Hermann A. Brück held "by far the most attractive astronomical post in the United Kingdom", that of being jointly Astronomer Royal for Scotland and Regius Professor of Astronomy at the University of Edinburgh. He has written a delightful book which will appeal to a wide variety of readers.

The story divides easily into three parts, the first centring around Calton Hill for which William Playfair designed an observatory modelled on the Greek Temple of the Winds. This replaced the Old Observatory on the same hill, which had been used by the Astronomical Institution of Edinburgh, a society founded in 1811, and the first British society whose activities were entirely devoted to the pursuit of astronomy. Their aim was to establish an observatory which could be used both for scientific research and for popular observation. In 1785 Robert Blair was appointed as the first professor of astronomy and this was followed in 1822 by King George IV being "graciously pleased to permit the Observatory on Calton Hill to be styled The Royal Observatory". In 1834 the Astronomical Institution transferred the use of the Observatory to the University on the condition that the Government of the day appointed an Observer who would be titled Astronomer Royal for Scotland and

would also occupy the Chair of Astronomy at the University of Edinburgh. Thomas Henderson was appointed and was followed by Charles Piazzi Smyth who dominated Edinburgh astronomy between 1846 and 1888.

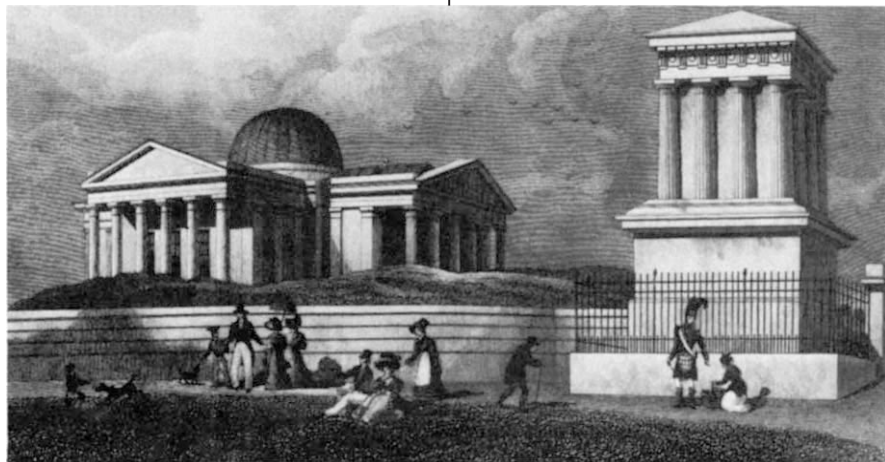
Smyth was one of the most fascinating personalities in the scientific world during the Victorian era. He founded "mountain astronomy", realizing that height improved the effectiveness and precision of astronomical observations, immersed himself in pyramidology and ignored his university duties as there were "generally no students at all for so untoward, despised and poverty-stricken a subject in Scotland".

Things obviously had to change and Ralph Copeland, Smyth's successor, witnessed the building of a new observatory at Blackford Hill to the south of Edinburgh and the transfer to that observatory of the Dun Echt instruments which previously belonged to the 26th Earl of Crawford. Spectroscopic astrophysics flourished and under the successive leadership of Frank Dyson, Ralph Sampson and W.M.H. Greaves the observatory developed spectrophotometry and high-precision photographic photometry.

In 1957 Hermann Brück took over and in his 18 years supervised a great expansion of the observatory, the numbers of staff growing from less than ten on his arrival to well over a hundred on his retirement. Research in Edinburgh led to the development of autoguider systems, photoelectric exposure control for spectroscopes, ultraviolet observatories in space, satellite tracking and the GALAXY and COSMOS machines which can automatically scan photographic plates and measure the positions and brightness of stars and galaxies.

Brück writes that his "years on Blackford Hill have been extremely happy ones", a happiness which echoes through his story. There can be few better advertisements for astronomy, or for Edinburgh as a place to pursue the subject. □

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Calton Hill in the 1820s, adorned by the New Observatory and Playfair's Monument.

Teleki to Leakey

Andrew Hill

Koobi Fora: Researches into Geology, Palaeontology, and Human Origins. Volume 2 The Fossil Ungulates: Proboscidea, Perissodactyla, and Suidae.
 Edited by J.M. Harris.
Oxford University Press: 1983. Pp.320. £45, \$95.

IT WAS less than a hundred years ago that Europeans first looked upon Lake Turkana, when Count Samuel Teleki von Szek and his fellow traveller Ludwig von Hohnel came across it on 6 March 1888. "An entirely new world was spread before our astonished eyes", wrote von Hohnel on seeing the huge expanse of water in the deserts of northern Kenya.

After Teleki few Westerners visited the lake. Sir Vivian Fuchs led an expedition there in the 1930s, and in 1943 Louis Leakey was told of the existence of fossils by a District Commissioner, but was denied access at that time. It was left to his son Richard to rediscover the palaeontological potential of the area in 1967. He began the Koobi Fora Research Project in 1968 and it still continues. While it may be hyperbolic to suggest an entirely new world has been spread before our astonished eyes as a result, certainly the research of the Project has changed our view of palaeoanthropology in significant ways.

In 1968 only three specimens of early hominid were known from Kenya. The Koobi Fora expedition has changed that, for a great deal of material has come from Lake Turkana, most of it discovered by an expert group of Kenyan fossil prospectors led by Kamoya Kimeu. Now the list of hominid fossils exceeds 150 for Koobi Fora alone, and the project has spawned as many publications. This monograph, edited and largely written by John Harris, is the most recent of them. It is dedicated to the memory of the late Sonia Cole, the technical editor of this and the first Koobi Fora volume, who in many ways made notable contributions to palaeoanthropology in Africa.

Aside from the sheer abundance of hominid specimens, what are the main achievements of the Project? Koobi Fora is one of three large sites in eastern Africa that document the radiation of hominids between two and one million years ago. Olduvai in Tanzania provided the first evidence, an assortment of specimens comprising two new taxa, *Australo-*

pithecus boisei and the originally more controversial *Homo habilis*. It also produced the earliest complete assemblages of lithic culture. Work near the Omo River in southern Ethiopia resulted in important collections of vertebrate fossils, and a series of volcanic tuffs through the succession made it possible to establish a reliable temporal framework by potassium-argon dating. Although numerically abundant, the hominid finds were not spectacular, as sedimentary conditions in that area favoured mainly the preservation of isolated teeth.

Koobi Fora provided more complete specimens. Amongst them are good examples of *Australopithecus boisei*, enabling us to appreciate the high degree of sexual dimorphism and range of variation in this interesting creature. There are specimens that appear to confirm the reality of *Homo habilis*, and yet others that probably represent a third species. This might explain some of the earlier interpretative problems within the Olduvai sample. Another step was the recognition at Koobi Fora of the great antiquity in Africa of *Homo erectus*. This is the species generally thought to be immediately ancestral to our own, and is also the first hominid found outside Africa. Most specimens from the Far East and Europe, though, are more recent than one million years, whereas some from Koobi Fora are half-a-million years older. This makes it possible that even *Homo erectus* originated in Africa rather than elsewhere.

A theory being pushed with some vigour until a few years ago was that once hominids and material culture were established there was room ecologically for only one species. The finding of *Homo erectus* at this level, where it was contemporary with at least *Australopithecus boisei*, successfully demolished that notion. Hominid post-cranial bones were also found, resulting in a better understanding of locomotion in these early groups.

A variety of other research has been conducted under the auspices of the expedition. Some of this is general and basic, for example that on evolutionary theory using the excellent record of fossil freshwater gastropods. Other work, such as the investigations of the geology, palaeoecology and taphonomy of recent and fossil bone assemblages, is essential to understanding the environmental and time context of the hominids. Some deals fairly directly with hominid activities. Glynn Isaac, who with Richard Leakey is the Project's co-director and co-editor of this Koobi Fora monograph series, has led a team of archaeologists which has described two successive patterns of early stone tool technology that comprise distinctive facies within the Oldowan Industrial Complex. The team has also done much valuable work on principles of site interpretation.

Although the stone tools and the bits and pieces of old human skeletons form the

glamorous treasures of palaeoanthropology, over 6,000 fossils of a wide range of other vertebrates have also been collected. These span the period from four to one million years ago, and are intrinsically important for what they tell us about the origins and evolution of certain taxonomic groups world wide, and also for their contribution to understanding the environments and ecology in which the hominids evolved. John Harris is the coordinator of palaeontology in the Project, and the present volume is the first of two dealing with the fossil ungulates. It is not designed for casual reading. Five of the seven chapters are detailed taxonomic treatments of the Deinotheriidae, Elephantidae (by Michel Beden), Rhinocerotidae, Equidae (by Vera Eisenmann), and Suidae, with complete listings and field locations of specimens. An introductory chapter gives the history of exploration and stratigraphical interpretation; a concluding one discusses correlation with other sites, biozonation and environmental change.

Correlation is one of the crucial questions to which the palaeontological information relates. The initial radiometric dating at Koobi Fora led to the impression that the artefacts there, and the fossil material belonging to the genus *Homo*, were the oldest in the world. Palaeoanthropologists traditionally have a proprietary attitude to their finds, and if they can possess the oldest they are in a very attractive position. It is attractive for publicity and therefore funding if nothing else, and so is a position they are understandably loath to give up. Such resistance was shown at Koobi Fora. Evidence was accumulating from the fauna which made clear an apparent time disjunction between there and other sites, particularly the Omo, only a few tens of kilometres distant. A number of ingenious explanations were devised to account for this disparity, before fresh dating, and more detailed work on the quite complicated stratigraphy, showed that earlier correlations were simply wrong. The hominids and artefacts are comparable in age to the oldest of their kind found elsewhere in East Africa, such as those from Olduvai. While slightly tarnishing the tinsel, this does little to reduce the area's scientific appeal.

Palaeontology is more than a description of biological events that just happen to have taken place in the past, and more than a simple narrative of phylogenetic histories. This present volume is an important work, and should in no sense be seen as a mere epitaph to these departed beasts. Rather it provides the basic data which, when taken with comparable information from other sites, could answer fundamental biological questions in evolution and ecology that neontology's perspective, limited as it is to extant creatures and communities, is too narrow to solve. □

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● *Pluto's Republic*, Sir Peter Medawar's foray into "the intellectual underworld", has recently been published in paperback. The book first appeared in 1982, and in his review (*Nature* 300, 118; 1982) Stuart Sutherland wrote of Medawar's "Johnsonian thump of common sense". Publisher of the paperback is Oxford University Press; price is £4.95.

Mathematics

Banach Bundles, Banach Modules and Automorphisms of C^* . Algebras. Research Notes in Mathematics, Vol. 92. By M.J. DUPRÉ and R.M. GILLETTE. Pitman: 1983. Pp.111. Pbk ISBN 0-273-08626-X. Np.

Birkhoff Interpolation. Encyclopedia of Mathematics and Its Applications, Vol. 19. Addison-Wesley: 1983. Pp.237. ISBN 0-201-13518-3. \$32.50.

Contributions to Nonlinear Partial Differential Equations. Research Notes in Mathematics, Vol. 89. By C. BARDOS et al. Pitman: 1983. Pp.284. Pbk ISBN 0-273-08595-6. Np.

Encyclopedia of Statistical Sciences, Vol. 3. S. KOTZ and N.L. JOHNSON (eds). Wiley: 1983. Pp.722. ISBN 0-471-05549-2. £75.50, \$105.75.

The Method of Differential Approximation. By Y.I. SHOKIN. Springer-Verlag: 1983. Pp.296. ISBN 3-540-12225-7/0-387-12225-7. DM 96, \$41.40.

Nonlinear Partial Differential Equations and Their Applications. Collège de France Seminar, Vol. 5. Research Notes in Mathematics, Vol. 93. By H. BREZIS and J.L. LIONS. Pitman: 1983. Pp.370. Pbk ISBN 0-273-08620-0. Np.

Similarity Solutions of Nonlinear Partial Differential Equations. Research Notes in Mathematics, Vol. 88. By L. DRESNER. Pitman: 1983. Pp.124. Pbk ISBN 0-273-08621-9. Np.

Space Science

Active Experiments in Space. Proceedings of an International Symposium held May 1983, Austria. European Space Agency: 1983. Pp.375. Pbk FF.140.

The Astronomical Almanac for the Year 1984. HMSO: 1983. Np.

Diffuse Matter in Galaxies. J. AUDOUZE et al. (eds). Reidel: 1983. Pp.262. ISBN 90-277-1626-9. Np.

Early Evolution of the Universe and Its Present Structure. G.O. ABELL and G.C. ARINI (eds). Reidel: 1983. Pp.536. Hbk ISBN 90-277-1653-6; pbk ISBN 90-277-1662-5. Hbk Dfl. 160, \$64; pbk Dfl. 85, \$34.

Glimpsing an Invisible Universe: The Emergence of X-Ray Astronomy. By R.F. HIRSH. Cambridge University Press: 1983. Pp.186. ISBN 0-521-25121-4. £20, \$39.50.

Guidance and Control 1983, Vol. 51: Advances in the Astronautical Sciences. E.J. BAUMAN and Z.W. EMSLEY (eds). American Astronautical Society: 1983. Pp.480. Hbk ISBN 0-87703-182-7; pbk ISBN 0-87703-183-5. Hbk \$60; pbk \$50.

The Near-Earth and Interplanetary Plasma, Vol. 1: General Properties and Fundamental Theory. By Ya. L. AL'PERT. Cambridge University Press: 1983. Pp.299. ISBN 0-521-24364-5. £35, \$69.50.

The Near-Earth and Interplanetary Plasma, Vol. 2: Plasma Flow, Plasma Waves and Oscillations. By Ya. L. AL'PERT. Cambridge University Press: 1983. Pp.259. ISBN 0-521-24601-6. £30, \$59.50.

Observational Basis for Velocity Fields in Stellar Atmosphere. R. STALIO (ed.). Observatorio Astronomico, Trieste: 1983. Pp.477. Np.

Software Engineering. Proceedings of ESA/ESTEC Seminar held October 1983, The Netherlands. European Space Agency: 1983. Pp.275. Pbk FF.140.

Solar and Stellar Magnetic Fields: Origins and Coronal Effects. Symposium No. 102. J.O. STENFLO (ed.). Reidel: 1983. Pp.564. Hbk ISBN 90-277-1619-6; pbk ISBN 90-277-1621-8. Np.

Spacecraft Vibration Testing Using Multi-Axis Hydraulic Vibration Systems. Proceedings of a Workshop held May 1983, The Netherlands. European Space Agency: 1983. Pp.173. Pbk FF.90.

Physics

Advances in Laser Spectroscopy. NATO Advanced Science Institutes Series, Vol. 95. F.T. ARECCHI, F. STRUMIA and H. WALTHER (eds). Plenum: 1983. Pp.506. ISBN 0-306-41355-8. \$69.50.

Advances in Superconductivity. NATO Advanced Science Institute Series, Vol. 100. B. DEAVER and J. RUVALDS (eds). Plenum: 1983. Pp.529. ISBN 0-306-41388-4. \$75.

Amorphous Magnetism and Metallic Magnetic Materials-Digest: A Survey of the Literature with a Complete Bibliography. By A.R. FERCHMIN and S. KOBE. Elsevier Biomedical/North-Holland: 1983. Pp.354. ISBN 0-444-86532-2. \$65.50, Dfl. 165.

Concise Encyclopedia of Solid State Physics. R.G. LERNER and G.L. TRIGG (eds). Addison-Wesley: 1983. Pp.311. Hbk ISBN 0-201-14204-X; pbk ISBN 0-201-14205-8. Hbk \$39.50; pbk \$22.95.

Elastic Media with Microstructure II: Three-Dimensional Models. Springer Series in Solid-State Sciences, Vol. 44. By I.A. KUNIN. Springer-Verlag: 1983. Pp.272. ISBN 3-540-12078-5/0-387-12078-5. DM 98, \$38.90.

The Elements of Mechanics. By G. GALLAVOTTI. Springer-Verlag: 1983. Pp.575. ISBN 0-387-11753-9/3-540-11753-9. DM 109, \$44.60.

A First Course in Fluid Dynamics. By A.R. PATERSON. Cambridge University Press: 1983. Pp.528. Hbk ISBN 0-521-25416-7; pbk ISBN 0-521-27424-9. Hbk £30, \$59.50; pbk £12.50, \$24.95.

Fourth Nordic Meeting on Nuclear Physics held August 1982, Fuglsø. Physica Scripta, Vol. T5. J. BONDORF and G. HAGEMANN (eds). Royal Swedish Academy of Sciences: 1983. Pp.223. Np.

The General Stokes' Theorem. By H. GRUNSKY. Pitman: 1983. Pp.113. ISBN 0-273-08510-7. £18.95.

Hyperfine Interactions of Radioactive Nuclei. Topics in Current Physics, Vol. 31. J. CHRISTIANSEN (ed.). Springer-Verlag: 1983. Pp.366. ISBN 3-540-12110-2/0-387-12110-2. DM 84, \$36.20.

Introduction to the Fourier Transform and Pseudo-Differential Operators. By B.E. PETERSEN. Pitman: 1983. Pp.356. ISBN 0-273-08600-6. £36.

Ion Implantation: Equipment and Techniques. Springer Series in Electrophysics, Vol. 11. Proceedings of the 4th International Conference held September 1982, FRG. H. RYSEL and H. GLAWISCHNIG (eds). Springer-Verlag: 1983. Pp.556. ISBN 3-540-12491-8/0-387-12491-8. DM 90, \$38.80.

Nonlinear Methods of Spectral Analysis. Topics in Applied Physics, Vol. 34. S. HAYKIN (ed.). Springer-Verlag: 1983. Pp.263. Pbk ISBN 3-540-123865/0-387-12386-5. DM 58, \$23.10.

Nordic Conference on Surface Science held August 1982, Tampere. Physica Scripta, Vol. T4. M. PESSA and R. NIEMINEN (eds). Royal Swedish Academy of Sciences: 1983. Pp.210. Np.

Point Defects in Semiconductors II: Experimental Aspects. Springer Series in Solid-State Sciences, Vol. 35. By J. BOURGOIN and M. LANNON. Springer-Verlag: 1983. Pp.295. ISBN 3-540-11515-3/0-387-11515-3. DM 82, \$35.40.

Process and Device Simulation for MOS-VLSI Circuits. P. ANTognetti et al. (eds). Martinus Nijhoff: 1983. Pp.632. ISBN 90-247-2689-1. Dfl. 180, \$78.50.

Production and Physics of Highly Charged Ions. Proceedings of an International Symposium held June 1982, Stockholm. Physica Scripta, Vol. T3. L. LLLJEBY (ed.). Royal Swedish Academy of Sciences: 1983. Pp.256. Np.

Quantum Chromodynamics: An Introduction to the Theory of Quarks and Gluons. By F.J. YNDURAIN. Springer-Verlag: 1983. Pp.227. ISBN 0-387-11752-0/3-540-11752-0. DM 78, \$31.

The Quest for Quarks. By B. McCUSKER. Cambridge University Press: 1983. Pp.160. ISBN 0-521-24850-7. £7.95, \$14.95.

Statistical Physics and the Atomic Theory of Matter From Boyle and Newton to Landau and Onsager. By S.G. BRUSH. Princeton University Press: 1983. Pp.356. Hbk ISBN 0-691-08325-8. Hbk £38.70; pbk £12.70.

Statistical Physics I: Equilibrium Statistical Mechanics. Springer Series in Solid-State Sciences, Vol. 30. By M. TODA, R. KUBO and N. SAITO. Springer-Verlag: 1983. Pp.249. ISBN 3-540-11460-2/0-387-11460-2. DM 82, \$33.90.

Techniques and Concepts of High-Energy Physics II. NATO Advanced Science Institute Series, Vol. 99. T. FERBEL (ed.). Plenum: 1983. Pp.358. ISBN 0-306-41385-X. \$49.50.

Topics in Ocean Physics. Proceedings of the International School of Physics Course held July 1980, Villa Monastero. A.R. OSBORNE and P.M. RIZZOLI (eds). Elsevier Biomedical/North-Holland: 1983. Pp.550. ISBN 0-444-86160-2. \$98, Dfl. 255.

Chemistry

Advanced Organic Chemistry, 2nd Edn. Part B: Reactions and Synthesis. By F.A. CAREY and R.J. SUNDBERG. Plenum: 1983. Pp.650. Hbk ISBN 0-306-41088-5; pbk ISBN 0-306-41199-7. Hbk \$59.50; pbk \$16.95.

Chemical Thermodynamics. By P.A. ROCK. Oxford University Press/University Science Books: 1983. Pp.548. ISBN 0-19-855712-5/0-935702-12-1. £22.50.

Chemists. The Biographical Dictionary of Scientists. D. ABBOTT (ed.). Blond Educational: 1983. Pp.203. ISBN 0-584-70000-8. £10.95.

Derivatives of Hydrazine and Other Hydronitrogens Having N-N Bonds. By P.A.S. SMITH. Benjamin/Cummings: 1983. Pp.335. ISBN 0-8053-8902-4. \$49.95.

Design 1982. The Institution of Chemical Engineers Symposium Series No. 76. Pergamon: 1983. Pp.381. ISBN 0-08-028773-5. £31.50, \$63.

Molecular Ions: Spectroscopy, Structure and Chemistry. T.A. MILLER and V.E. BONDYBEY (eds). Elsevier Biomedical/North-Holland: 1983. Pp.278. ISBN 0-444-86717-1. \$48, Dfl. 125.

Organic Chemistry in Colour. By P.F. GORDON and P. GREGORY. Springer-Verlag: 1983. Pp.322. ISBN 3-540-11748-2/0-387-11748-2. DM 178, \$70.70.

Problems in Quantum Chemistry. By P. JØRGENSEN and J. ØDDERSHEDE. Addison-Wesley: 1983. Pp.286. ISBN 0-201-05486-8. \$26.95.

Progress in Crystal Growth and Characterization, Vol. 5. B.R. PAMPLIN (ed.). Pergamon: 1983. Pp.420. ISBN 0-08-031011-7. £78, \$141.

Progress in Reaction Kinetics, Vol. 11. K.R. JENNINGS and R.B. CUNDALL (eds). Pergamon: 1983. Pp.274. ISBN 0-08-030040-5. £60, \$103.

Progress in Solid State Chemistry, Vol. 14. G.M. ROSENBLATT and W.L. WORRELL (eds). Pergamon: 1983. Pp.312. ISBN 0-08-030998-4. £66, \$120.

Steric Effects in Drug Design. Topics in Current Chemistry, Vol. 114. M. CHARTON and I. MOTOC (eds). Springer-Verlag: 1983. Pp.161. ISBN 3-540-12398-9/0-387-12398-9. DM 76, \$30.20.

Computer Science

Advances in Computers, Vol. 22. M.C. YOVITS (ed.). Academic: 1983. Pp.377. ISBN 0-12-012122-0. \$60.

Basic Microcomputing and Biostatistics: How to Program and Use Your Microcomputer for Data Analysis in the Physical and Life Sciences, including Medicine. By D.W. ROGERS. Humana: 1983. Pp.304. ISBN 0-89603-015-6. \$39.50 (US), \$49.50 (elsewhere).

Biodata Handling with Microcomputers. By R.B. BARLOW. Elsevier Biomedical/North-Holland: 1983. Pp.261. Pbk ISBN 0-444-80539-7. \$30, £18.

Microcomputers in Plain English. By B.D. STRONG. David & Charles: 1983. Pp.112. ISBN 0-7153-8509-7. £5.95.

Earth Sciences

Air-Sea Exchange of Gases and Particles. P.S. LISS and W.G.N. SLINN (eds). Reidel: 1983. Pp.561. ISBN 90-277-1610-2. Dfl. 160, \$69.50.

Changing Climate: Report of the Carbon Dioxide Assessment Committee. National Academy Press: 1983. Pp.496. Pbk ISBN 0-309-03425-6. \$29.50.

Chemical Oceanography, Vol. 8. J.P. RILEY and R. CHESTER (eds). Academic: 1983. Pp.398. ISBN 0-12-588608-X. £45, \$75.

Coastal Oceanography. NATO Conference Series, Vol. 11. H.G. GADE, A. EDWARDS and H. SVENDSEN (eds). Plenum: 1983. Pp.582. ISBN 0-306-41374-4. \$79.50.

Continental Basalts and Mantle Xenoliths. C.J. HAWKESWORTH and M.J. NORRIS (eds). Shiva, Cheshire: 1983. Pp.272. Hbk ISBN 0-906812-33-X; pbk ISBN 0-906812-34-8. Hbk £25; pbk £12.50.

Dictionary of Petrology. By S.I. TOMKEIEFF. Wiley: 1983. Pp.680. ISBN 0-471-10159-1. £49.50, \$97.50.

Geochemistry of Sedimentary Ore Deposits. By J.B. MAYNARD. Springer-Verlag: 1983. Pp.305. ISBN 0-387-90783-1/3-540-90783-1. DM 69, \$27.40.

International Minerals: A National Perspective. A.F. AGNEW (ed.). Westview: 1983. Pp.164. ISBN 0-86531-622-8. Np.

Large-Scale Dynamical Processes in the Atmosphere. B. HOSKINS and R. PEARCE (eds). Academic: 1983. Pp.390. ISBN 0-12-356680-0. £33, \$54.50.

Mega-Geomorphology. By R. GARDNER and H. SCOGING. Oxford University Press: 1983. Pp.240. ISBN 0-19-823244-6. £20.

Migmatites, Melting and Metamorphism. Proceedings of the Geochemical Group of the Mineralogical Society. M.P.ATHERTON and C.D. GRIBBLE (eds). Shiva, Cheshire: 1983. Pp.326. Hbk ISBN 0-906812-26-7; pbk ISBN 0-906812-25-9. Hbk £25; pbk £12.50.

The Mountains of Northeastern Tasmania: A Study of Alpine Geomorphology. By N. CAINE. A.A. Balkema: 1983. Pp.200. ISBN 90-6191-289-X. £15.50.

Noble Gas Geochemistry. By M. OZIMA and F.A. PODOSEK. Cambridge University Press: 1983. Pp.367. ISBN 0-521-23939-7. £40, \$79.50.

Optimal Seismic Deconvolution: An Estimation-Based Approach. By J.M. MENDEL. Academic: 1983. Pp.254. ISBN 0-12-490780-6. \$37.50.

Palaeomagnetism: Principles and Applications in Geology, Geophysics and Archaeology. By D.H. TARLING. Chapman & Hall: 1983. Pp.379. Hbk ISBN 0-412-23920-5; pbk ISBN 0-412-25100-0. Hbk £25; pbk np.

Technology

Advances in Nuclear Science and Technology. Vol. 15. J. LEWINS and M. BECKER (eds). Plenum: 1983. Pp.406. ISBN 0-306-41392-2. \$57.50.

Hydrogen: Energy Vector of the Future. Graham & Trotman: 1983. Pp.230. ISBN 0-86010-451-6. £22, \$41.

Modern Chlor-Alkali Technology. Vol. 2. C. JACKSON (ed.). Ellis Horwood/Halsted: 1983. Pp.389. ISBN 0-85312-525-2/0-470-27471-9. £35.

Remote Sensing Applications for Environmental Studies. Proceedings of the EARSeL/ESA Symposium held April 1983, Brussels. European Space Agency: 1983. Pp.291. Pbk FF.170.

Thin Film Device Applications. By K.L. CHOPRA and I. KAUR. Plenum: 1983. Pp.300. ISBN 0-306-41297-7. \$42.50.

VLSI Electronics Microstructure Science. Vol. 6: Materials and Process Characterization. N.G. EINSRUCH and G.B. LARRABEE (eds). Academic: 1983. Pp.601. ISBN 0-12-234106-6. \$79.

Applied Biological Sciences

Advances in Cancer Research. Vol. 39. G. KLEIN and S. WEINHOUSE (eds). Academic: 1983. Pp.343. ISBN 0-12-006639-4. \$46.

Analytical Profiles of Drug Substances. Vol. 12. K. FLOREY (ed.). Academic: 1983. Pp.735. ISBN 0-12-260812-7. \$47.

Biofeedback and Family Practice Medicine. W.H. RICKLES et al. (eds). Plenum: 1983. Pp.244. ISBN 0-306-41386-8. \$29.50.

Biological Aspects of Alzheimer's Disease. Banbury Report 15. R. KATZMAN (ed.). Cold Spring Harbor Laboratory: 1983. Pp.375. ISBN 0-87969-213-8. \$55 (US), \$66 (elsewhere).

Biotechnology, Chemical Feedstocks and Energy Utilization. By D.F. GIBBS and M.E. GREENHALGH. Frances Pinter: 1983. Pp.116 + appendix. ISBN 0-86187-346-7. £16.50.

Chemical Mutagens: Principles and Methods of Their Detection. Vol. 8. F.J. de SERRES (ed.). Plenum: 1983. Pp.386. ISBN 0-306-41336-1. \$45.

Clinical and Biological Aspects of Peripheral Nerve Diseases. Neurology and Neurobiology, Vol. 4. Proceedings of the Symposium held September 1982, Italy. L. BATTISTIN, G.A. HASHIM and A. LAJTHA (eds). Alan R. Liss: 1983. Pp.402. ISBN 0-8451-2703-9. £37.

Compartmental Modeling and Tracer Kinetics. Lecture Notes in Biomathematics, Vol. 50. By D.H. ANDERSON. Springer-Verlag: 1983. Pp.302. Pbk ISBN 3-540-12303-2/0-387-12303-2. DM 44, \$17.50.

Computer-Aided Electromyography. Progress in Clinical Neurophysiology, Vol. 10. J.E. DESMEDT (ed.). Karger: 1983. Pp.334. ISBN 3-8055-3748-4. SwFr. 165, DM 198, \$99.

Culture of Animal Cells: A Manual of Basic Technique. By R.I. FRESHNEY. Alan R. Liss: 1983. Pp.310. ISBN 0-8451-0223-0. £38.

Diabetes, Obesity and Hyperlipidemias II. G. CREPALDI, P.J. LEFÈVRE and D.J. GALTON (eds). Academic: 1983. Pp.552. ISBN 0-12-195480-3. £25, \$42.

The Drug Handbook 1983-4. By P. TURNER and G.N. VOLANS. Macmillan Press, London: 1983. Pp.168. Pbk ISBN 0-333-34974-1. £4.95.

Ecological Aspects of Used-Water Treatment. Vol. 2: Biological Activities and Treatment Processes. C.R. CURDS and H.A. HAWKES (eds). Academic: 1983. Pp.308. ISBN 0-12-199502-X. £32, \$55.

Female Stress Incontinence. Contributions to Gynecology and Obstetrics, Vol. 10. U. ULMSTEN (ed.). Karger: 1983. Pp.72. ISBN 3-8055-3665-8. SwFr. 55, DM 66, \$33.

Food Chains to Biotechnology. Selected Topics in Biology. By J.B. LAND and R.B. LAND. Nelson: 1983. Pp.208. Pbk ISBN 0-17-448088-1. £4.50.

Food Microbiology: Advances and Prospects. The Society for Applied Bacteriology Symposium Series No. 11. T.A. ROBERTS and F.A. SKINNER (eds). Academic: 1983. Pp.416. ISBN 0-12-589670-0. £29.95, \$49.95.

Sleep/Wake Disorders: Natural History, Epidemiology, and Long-Term Evolution. G. GUILLEMINAULT and E. LUGARESI (eds). Raven: 1983. Pp.302. ISBN 0-89004-906-8. \$58.50.

Techniques in Immunocytochemistry. Vol.2. G.R. BULLOCK and P. PETRUSZ (eds). Academic: 1983. Pp.300. ISBN 0-12-140402-1. £25, \$41.50.

Ultrasound Interactions in Biology and Medicine. R. MILLNER, E. ROSENFELD and U. COBET (eds). Plenum: 1983. Pp.216. ISBN 0-306-41367-1. \$37.50.

Upgrading Waste for Feeds and Food. By D.A. LEDWARD, A.J. TAYLOR and R.A. LAWRIE. Butterworths: 1983. Pp.321. ISBN 0-408-10837-1. £34.

Urologic Cancer: Chemotherapeutic Principles and Management. Recent Results in Cancer Research, Vol.85. F.M. TORTI (ed.). Springer-Verlag: 1983. Pp.151. ISBN 3-540-12163-3/0-387-12163-3. DM 98, \$42.30.

Psychology

Advances in Clinical Child Psychology. Vol.6. B.B. LAHEY and A.E. KAZDIN (eds). Plenum: 1983. Pp.328. ISBN 0-306-41330-2. \$39.50.

CNS Receptors: From Molecular Pharmacology to Behaviour. Advances in Biochemical Psychopharmacology, Vol.37. P. MANDEL and F.V. DeFEUDIS (eds). Raven: 1983. Pp.528. ISBN 0-89004-827-4. \$60.

Current Issues in Clinical Psychology. Vol.1. E. KARAS (ed.). Plenum: 1983. Pp.301. ISBN 0-306-41362-0. \$42.50.

The Psychology of Cognition. 2nd Edn. By G. COHEN. Academic: 1983. Pp.277. Hbk 0-12-178760-5; pbk ISBN 0-12-178762-1. Hbk np; pbk £8.40, \$16.

Research on the Viral Hypothesis of Mental Disorders. Advances in Biological Psychiatry, Vol.12. P.V. MOROZOV (ed.). Karger: 1983. Pp.175. Pbk ISBN 3-8055-3706-9. SwFr. 91, DM 109, \$54.50.

History of Science

L'esprit des Protéines: Histoire et Philosophie Biochimiques. By C. DEBRU. Hermann, Paris: 1983. Pp.368. Pbk ISBN 2-7056-5952-8. FF 96.

Geometry and Algebra in Ancient Civilizations. By B.L. VAN DER WAERDEN. Springer-Verlag: 1983. Pp.223. ISBN 3-540-12159-5/0-387-12159-5. DM 78, \$32.30.

History of Staining. 3rd Edn. By G. CLARK and F.H. KASTEN. Williams & Wilkins: 1983. Pp.304. ISBN 0-683-01705-5. £22.25.

Raymond Arthur Dart: A Pictorial Profile. By F. WHEELHOUSE. Transpareon Press, Sydney: 1983. Pp.113. ISBN 0-908021-046. Np.

Anthropology

Ancient Sedimentary Environments and the Habitats of Living Organisms. By J.C. GALL. Springer-Verlag: 1983. Pp.220. ISBN 3-540-12137-4/0-387-12137-4. DM 60, \$24.80.

Koobi Fora, Vol.2: The Fossil Ungulates. J.M. HARRIS (ed.). Oxford University Press: 1983. Pp.321. ISBN 0-19-857398-7. £45.

Man, A Geomorphological Agent: An Introduction to Anthropogenic Geomorphology. By D. NIR. Reidel: 1983. Pp.165. ISBN 90-277-1401-0. Np.

Quaternary Coastlines and Marine Archaeology: Towards the Prehistory of Land Bridges and Continental Shelves. P.M. MASTERS and N.C. FLEMMING (eds). Academic: 1983. Pp.660. ISBN 0-12-479250-2. £25, \$42.

The Search for our Beginning: An Enquiry, Based on Meteorite Research, into the Origin of our Planet and its Life. By R. HUTCHISON. Oxford University Press/British Museum (Natural History): 1983. Pp.164. ISBN 0-19-858505-5/0-190520435-5/0-565-00851-X. £7.95.

The Shanidar Neandertals. By E. TRINKAUS. Academic: 1983. Pp.502. ISBN 0-12-700550-1. \$47.50.

General

Acid Rain: A Review of the Phenomenon in the EEC and Europe. Graham & Trotman: 1983. Pp.159. ISBN 0-86010-501-6. £12.50.

Advanced Synergetics: Instability Hierarchies of Self-Organizing Systems and Devices. Springer Series in Synergetics, Vol.20. By H. HAKEN. Springer-Verlag: 1983. Pp.356. ISBN 3-540-12162-5/0-387-12162-5. DM 98, \$38.90.

Apples: A Guide to the Identification of International Varieties. By J. BULTITUDE. Macmillan, London: 1983. Pp.323. ISBN 0-333-34971-7. £27.50.

The Arid Lands: Their Use and Abuse. By R.L. HEATHCOTE. Longman: 1983. Pp.323. Pbk ISBN 0-582-30048-7. £7.95.

Betrayers of the Truth. By W. BROAD and N. WADE. Simon & Schuster: 1983. Pp.256. Pbk ISBN 0-671-49549-6. \$6.95.

Beyond Mind. By W.E.R. MONS. Rider, London: 1983. Pp.255. ISBN 0-09-153550-6. £6.95.

Biomedical Institutions, Biomedical Funding, and Public Policy. H.H. FUDENBERG (ed.). Plenum: 1983. Pp.212. ISBN 0-306-41231-4. \$29.50.

Birdwatch Around Scotland. By W.R. MITCHELL. Robert Hale: 1983. Pp.175. ISBN 0-7090-1248-9. £9.95.

Care of the Wild: First Aid for All Wild Creatures. By W.J. JORDAN and J. HUGHES. Rawson Associates: 1983. Pp.223. Hbk ISBN 0-89256-226-9; pbk ISBN 0-89256-240-4. Hbk \$13.95; pbk \$8.95.

Chemistry and World Food Supplies: The New Frontiers. Chemrawn II. L.W. SHEMILT (ed.). Pergamon: 1983. Pp.664. Hbk ISBN 0-08-029243-7; pbk ISBN 0-08-029242-9. Hbk £75, \$135; pbk £42, \$75.

The Classification of Finite Simple Groups. Vol.1: Groups of Noncharacteristic 2 Type. By D. GORENSTEIN. Plenum: 1983. Pp.487. ISBN 0-306-41305-1. \$59.50.

Cosmic Crystals. By R. BONEWITZ. Turnstone Press: 1983. Pp.192. Pbk ISBN 0-85500-205-0. £3.95.

Country Man on the Moors. By J.C. ATKINSON. Oxford University Press: 1983. Pp.160. Pbk ISBN 0-19-281414-1. £2.50.

The Culture of Technology. By A. PACEY. Basil Blackwell: 1983. Pp.210. ISBN 0-631-13236-8. £15.

The Dangers of New Weapon Systems. W. GUTTERIDGE and T. TAYLOR (eds). Macmillan Press, London: 1983. Pp.241. ISBN 0-333-35018-9. £25.

Dark-Time World: Watching and Photographing Wildlife at Night. By E. BARTLETT. Robert Hale: 1983. Pp.176. ISBN 0-7090-0996-8. £9.50.

Dear Lord Rothschild: Birds, Butterflies and History. By M. ROTHSCHILD. Hutchinson: 1983. Pp.398. ISBN 0-09-153740-1. £14.95.

Decentralizing Electricity Production. H.J. BROWN (ed.). Yale University Press: 1983. Pp.259. ISBN 0-300-02569-6. £27.

The Demon in the Aether: The Story of James Clerk Maxwell. By M. GOLDMAN. Paul Harris, Edinburgh: 1983. Pp.224. ISBN 0-86228-026-5. £18.

Dictionary of Scientific and Technical Terms. 3rd Edn. S.P. PARKER (ed.). McGraw-Hill: 1983. Pp.1,846. ISBN 0-07-045269-5. \$70.

Energy Storage, Compression and Switching. Vol.2. V. NARDI, H. SAHLIN and W.H. BOSTICK (eds). Plenum: 1983. Pp.1,067. ISBN 0-306-41014-1. \$125.